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Comparative proteomic study of the venom of the piscivorous cone snail *Conus consors*

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ABSTRACT

In the context of an exhaustive study of the piscivorous cone snail *Conus consors*, we performed an in-depth analysis of the intact molecular masses that can be detected in the animal's venom, using MALDI and ESI mass spectrometry. We clearly demonstrated that, for the venom of this species at least, it is essential to use both techniques in order to obtain the broadest data set of molecular masses. Only 20% of the total number of molecules detected were found in both mass lists. The two data sets were also compared in terms of mass range and relative hydrophobicity of the components detected in each. With a view to an extensive analysis of this venom's proteome, we further performed a comparative study by ESI-MS between venom obtained after classical dissection of the venom duct *versus* venom obtained by milking live animals. Surprisingly, although many fewer components were found in the milked venom than in the dissected venom, ~50% of those found had not been seen in the dissected venom. Several questions raised by these observations are discussed. With regards to the current knowledge of the cone snail venom composition, our results emphasize the complementary nature of the mass spectrometry methods and of the two techniques used in venom collection.

1. Introduction

Animal venoms are highly complex mixtures of biologically active compounds. These biomolecules have been tailor-made by millions of years of evolution to endow their possessors with the means to carry out the specific offensive and defensive tasks needed for their survival [1]. Venom molecules were thus gradually modified to match a multitude of highly specific targets, hence their unique pharmacological properties. Thanks to the resemblance of these targets to mammalian receptors, a remarkable number of venom components has been successfully developed as new research tools and therapeutic drugs [2]. Unfortunately, despite the large number of venomous animals and the complexity of their venoms, only a tiny proportion (estimated to represent less than 0.1%) of venom components have been identified and characterized, and less than 1% of genetic information is available [3]. While this low percentage may reflect the sparseness of some

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venomous animals, it also stems from the bioactivity-guided research approaches traditionally implemented to find new bioactive molecules. Indeed, very often, a biological activity assay is the first step taken in the quest for new compounds, followed by the isolation and characterization of the native bioactive substance from natural libraries. This strategy is time consuming and requires large amounts of material. Nowadays, current mass spectrometry (MS) techniques can generate an abundance of valuable data not only in a very short period of time, but more importantly using much smaller sample amounts [4]. Through a structure-driven process and thanks to constant evolving biocomputing capacities, MS has become paramount not only for analytical purposes, but also for the rapid discovery and characterization of new components in the field of toxinology [5]. When used in combination with DNA sequencing from cDNA libraries or ESTs, a wealth of information on the venom gland components can be obtained [6–8].

Different types of mass spectrometers are used to untangle the complexity of venom mixtures and to rapidly produce a large amount of information, such as the molecular masses of intact components, the number of disulfide bridges, and primary sequences. Not only do the accuracy and sensitivity of these instruments now enable us to distinguish between species on the basis of venom composition (for quality control of individual venom batches or for taxonomic or phylogenic studies), but they can also reveal intersexual and other intraspecific variations [9–11]. Although MALDI-MS equipped with a time-of-flight (TOF) mass analyzer proves to be particularly well suited to study complex venom compositions, this technique suffers from a low dynamic range, ion suppression effects and poor resolution in the linear mode for high masses [12]. Furthermore, the introduction of an off-line RP-HPLC step with fraction collection and freeze-drying prior to the analysis by MALDI-TOF-MS and ESI-MS of isolated fractions adds an important increasing factor to mass detection [13,14]. Most of these studies also tend to prove that, in many cases, the number of components present in venoms is consistently underestimated. The real size and variability of individual venom proteomes remain an open question.

In this study, we propose two comparative analyses of complex venom mixtures that initiate an in depth venomic project of a marine venomous organism, namely *Conus consors* [3]. We first present a comparison between MALDI-MS and ESI-MS analyses of individual RP-HPLC fractions of this fish-hunting (piscivorous) cone snail venom to determine the extent of overlap and/or complementarities between these two techniques in terms of mass detection. We also evaluate to what extent a given technique is better suited for the detection of high *versus* low masses and hydrophilic *versus* hydrophobic components present in these specific venoms. So far, studies on cone snail venom have always relied on dissected material, with very few exceptions. However, a recent study has shown that the injected venom is significantly different to that of dissected preparations [15]. We therefore also propose a direct comparison of all the masses detected using ESI-MS, between on the one hand, dissected *C. consors* venom and on the other hand, venom milked from live specimens of the same species.

2. Materials and methods

Acetonitrile (ACN, from Fisher Scientific Ltd., Loughborough, UK), trifluoroacetic acid (TFA, from Pierce–Perbio, Lausanne, Switzerland) and formic acid (Acros Organics, Geel, Belgium) were of HPLC gradient grade or higher. Deionised water was purified using a Milli-Q system (Millipore Corp., Billerica, MA, USA). When needed, each solvent prepared was filtered and sonicated before use.

2.1. *Venom preparations*

All 25 specimens of *C. consors* used for this study were collected from one colony in the Chesterfield Islands (New Caledonia) in the frame of the CONFIELD scientific expedition in June 2007. The pool of crude venom, referred to as dissected venom (DV) further in this article, was obtained after dissection of 19 *C. consors* specimens following the previously described method [16]. The crude venom pool has been lyophilized and weighed 35.8 mg. The protein content of this pool was estimated at about 7 mg (20%). For proteomic analyses, the lyophilized dissected venoms pool was reconstituted at 1 mg/mL (protein content) in acidified water (0.1% TFA) and desalted using solid-phase extraction onto a Sep-Pak Vac 35 cc cartridge equilibrated in acidified water according to the manufacturer's instructions (Waters, Milford, MA, USA). Elution was performed with 70% ACN in acidified water and the eluate freeze-dried under vacuum in a SpeedVac concentrator (Thermo-Savant, Holbrook, NY, USA), then stored at −80 °C. The pool of milked venom (MV) was obtained by combining 67 individual milkings from a batch of 6 specimens kept alive in our aquariums. The milked venom was lyophilized and stored at −80 °C. Its total protein content was estimated at about 400 μg.

2.2. *RP-HPLC*

Reversed-phase high-performance liquid chromatography (RP-HPLC) was carried out using a Waters Alliance 2795 system equipped with a Waters 996 Photodiode Array Detector under control of the Waters Millenium^{32} 4.0 software (Waters, Milford, MA, USA). All fractionations were performed using a 218TP510 Protein and Peptide C_{18} RP column (10 mm internal diameter/250 mm length, from Vydac, Hesperia, CA, USA) with a gradient combining solvent A (0.1% TFA in water) and solvent B (90% ACN / 0.1% TFA in water). A flow rate of 2.0 mL/min was used with a gradient of 1% B per minute, starting from 100% of solvent A. UV detection of the fractions was carried out at 214 nm and fractions were collected manually. The dissected venom pool was subjected to 5 RP-HPLC runs and corresponding fractions were pooled. The milked venom pool was fractionated in a single RP-HPLC run. All final fractions were freeze-dried and stored at −80 °C.

2.3. *MALDI-MS*

MALDI-MS analyses were carried out on an Ultraflex TOF–TOF mass spectrometer operated in positive reflector and linear modes under control of the FlexControl 2.2 software (Bruker, Bremen, Germany). 2% of dissected venom fractions were dissolved in 10 μL of solvent (H_2O/ACN/TFA, 79.92:20:0.08, v/v/v).