Unravelling Conotoxin Folding and Molecular Diversity

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Predatory marine cone snails of the genus *Conus* synthesise a great diversity of disulfide-rich neurotoxic peptides commonly referred to as conotoxins. Conotoxins selectively target specific subtypes of receptors or ion channels throughout the nervous system. This characteristic has led to the wide use of conotoxins in ion channel research and their exploration and use as therapeutic agents. A number of conotoxins show promise as pharmaceutical agents, particularly as analgesics for the treatment of neuropathic pain. For example, the ω-conotoxin MVIIA, a calcium channel agonist isolated from *Conus magus*, has successfully completed phase III clinical trials and is now distributed as Ziconotide (Prialt) by Elan Pharmaceuticals, USA. Other conotoxins such as the Ca^{2+} agonist CVID and the χ-conotoxin MrIA targeting the norepinephrine transporter have reached various stages of clinical trials (1).

Cone snail venom is estimated to contain up to 2,000 different peptide species at any given time (2). This mixture of peptides appears to be highly dynamic and can vary dramatically at different stages of cone snail development (3) as well as displaying great intra- and inter-species variation. Collectively, it is conservatively estimated that over 100,000 conotoxins exist, each with potentially different bioactivities. Remarkably, this vast library of bioactive compounds contained within cone snail venom has been generated from a relatively small number of gene superfamilies (4). Conotoxins are translated as precursor proteins with an N-terminal signal sequence, an intermediate pro-region followed by the mature toxin at the C-terminus. Comparisons between the different gene superfamilies reveal high conservation of the primary amino acid sequence for the signal and pro-sequence while the mature toxin region undergoes hypermutation between a conserved pattern of cysteine residues (4). The venom repertoire is further extended through the addition of post-translational modifications, some of which are known to increase toxin potency (5) and/or aid in stabilising the three-dimensional structure of the molecule (6). Post-translational modifications in *Conus* include common alterations such as disulfide bond formation and C-terminal amidation, as well as unusual modifications, such as bromination of tryptophan, carboxylation of glutamate and isomerisation of L- to D-amino acids (7). Little is known about the mechanisms responsible for the generation of such a broad variety and density of post-translational modifications in *Conus*.

In order to deliver this complex cocktail of bioactive peptides, cone snails have evolved a highly complex venom apparatus consisting of a long convoluted duct for venom biosynthesis, a muscular bulb involved in venom movement and harpoon-like radula teeth responsible for venom delivery (**Fig. 1**). Following biosynthesis in the epithelial cells of the venom duct, the venom is packaged into granules and secreted into the lumen of the duct. Evidence of venom gland epithelial cell specialisation is emerging (8,9) and mRNA expression of different conotoxin superfamilies was shown to occur in distinct parts of the venom duct (10), suggesting further specialisation of distinct regions of the venom gland. Whether certain superfamilies or different toxins from within a superfamily interact with distinct enzymes in discrete regions of the venom duct is unknown. However, we believe it is highly probable and this remains a question that we address in our laboratories using a combination of proteomics, histology and molecular biology.

A fundamental question we wanted to address was whether venom diversity observed between different species was reflected in the protein composition of their respective venom glands. We undertook a comprehensive comparison of venom composition from two *Conus* species, *C. novaehollandiae* and *C. victoriae*, which highlighted the diversity in their venom composition (**Fig. 2**) (9). Of note, only a 12% overlap in venom composition was observed, consistent with other studies of both inter- and intra-specific venom composition (2). We next examined the proteome

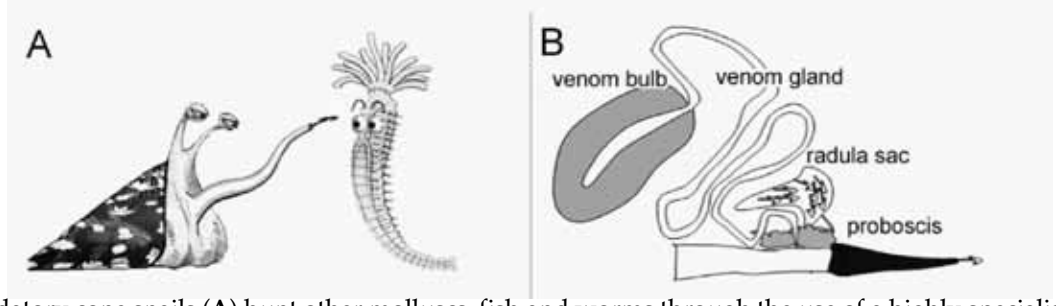

Fig. 1. Predatory cone snails (**A**) hunt other molluscs, fish and worms through the use of a highly specialised venom apparatus (**B**) composed of a muscular venom bulb, venom gland, radula sac and a harpoon like proboscis.

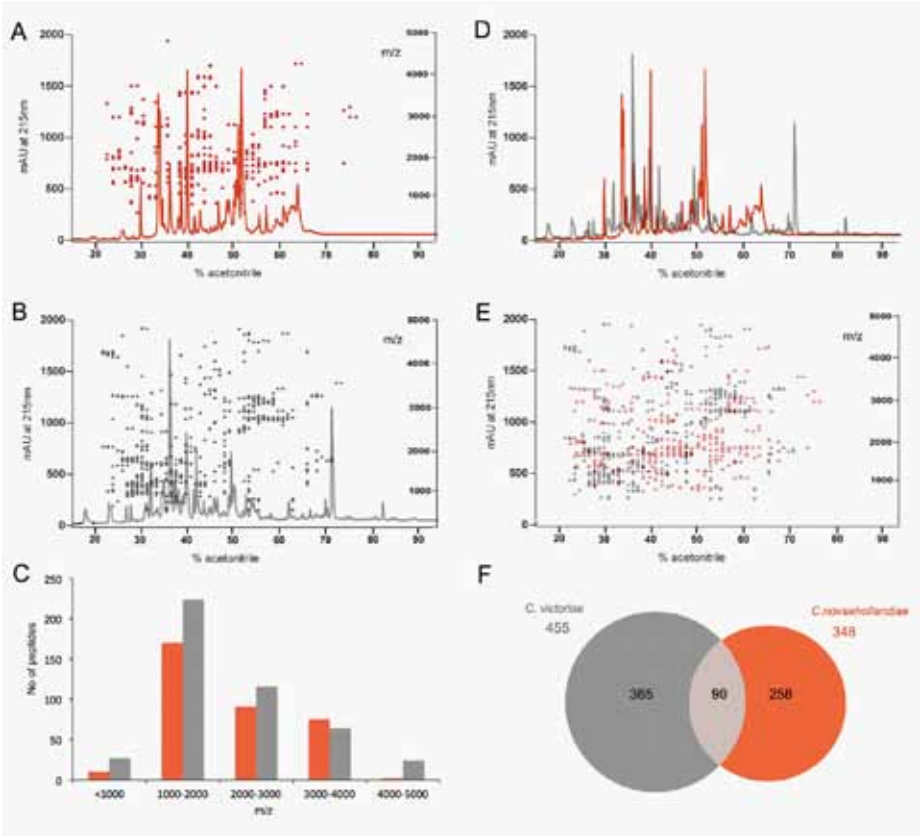

Fig. 2. Analysis and variation of the venom peptidome between *Conus* species. Reversed-phase chromatograms of venom extracted from (**A**) *C. novaehollandiae* and (**B**) *C. victoriae* overlaid with the peptide profiles obtained by MALDI-TOF analysis (m/z on the right y-axis). (**C**) Bar graph showing mass distribution of peptides. Overlap of (**D**) reversed-phase chromatograms and (**E**) peptide profiles illustrating interspecies differences in the venom peptidome. A total of 365 and 258 peptides with unique masses were identified in *C. victoriae* (grey) and *C. novaehollandiae* (orange) respectively with 90 common peptides identified between the two species (**F**). Reprinted (adapted) with permission from Safavi-Hemami *et al.* Specialisation of the venom gland proteome in predatory cone snails reveals functional diversification of the conotoxin biosynthetic pathway. *J. Proteome. Res.* **10**, 3904-3919. Copyright 2011 American Chemical Society.

of the venom gland from each species which showed extensive differences in the abundance of various protein species, as well as remarkable diversity in the expression of protein isoforms (**Fig. 3**). Our data clearly demonstrate that venom diversity goes hand in hand with venom gland proteome diversity. In contrast, the proteome of the venom bulb, a specialised organ responsible for venom ejection, was very similar between the two *Conus* species (compare **Fig. 3A** with **3B**). An analysis of protein class expression reveals functional similarities of each venom gland proteome from the two species, despite extensive differences in protein composition. This is consistent with the function of the gland and reflects the common requirements for venom biosynthesis. Thus it appears that as venoms have diversified between species, so too has the venom gland proteome, highlighting a novel mechanism of venom diversification.

Conotoxins with the same disulfide scaffold can display an array of folding properties, suggesting that neither the cysteine pattern nor the primary amino acid sequence are key folding determinants *in vitro* (11). Discrepancies between *in vivo* and *in vitro* folding of conotoxins are evident (11), with toxins that are difficult to synthesise chemically often being highly abundant in the snail's venom gland (12,13). These findings strongly suggest that the three-dimensional structure conotoxins adopt *in vivo* is highly regulated and determined by specific interactions with folding enzymes and molecular chaperones. Oxidation and isomerisation of disulfide bonds in the endoplasmic reticulum (ER) of *Conus* is now known to be catalysed by protein disulfide isomerase (PDI) (8,14), one of the most abundant soluble proteins in the venom gland of *Conus* (15,16) (**Fig. 3**). PDI was first characterised as a chaperone of reduction, oxidation and isomerisation of disulfide bonds (17). Since then, various other functions have been described for this ubiquitous protein. PDI catalyses folding of proteins with no disulfides and is a subunit of prolyl 4-hydroxylase (P4H), the enzyme that catalyses hydroxylation of proline residues (18), another common post-translational modification in *Conus* (5). Findings from our laboratory (15) suggest that the venom duct harbours multiple isoforms of PDI. We hypothesise that these isoforms have been recruited into the ER to interact with distinct conotoxin superfamilies in discrete regions of the venom gland. This diversification of PDI isoforms is reflected in the complex proteomes of the venom glands from different species of *Conus* (**Fig. 3**). Furthermore, recent data obtained in our laboratory suggest that PDI, as well as other enzymes important for conotoxin biosynthesis, are organised into multi-protein complexes. Folding of the μ-conotoxin GIIIA, a peptide with three hydroxyprolines and three disulfide bonds, was significantly accelerated in the presence of *Conus* peptidyl-prolyl isomerase (PPI) (19). Recent data from our laboratory further indicate that folding of μ-GIIIA and α-GI, a conotoxin with two disulfides and one proline, is most efficient in the presence of both PDI and PPI. Recently, a protein complex containing PDI and PPI was identified in a human hepatoma cell line (20). We have recently identified similar multi-enzyme complexes in the ER of the epithelial cells of the *Conus* venom duct (Safavi-Hemami *et al.*, unpublished data). Thus a picture of conotoxin folding is emerging that involves the co-evolution of folding enzymes and toxin species with

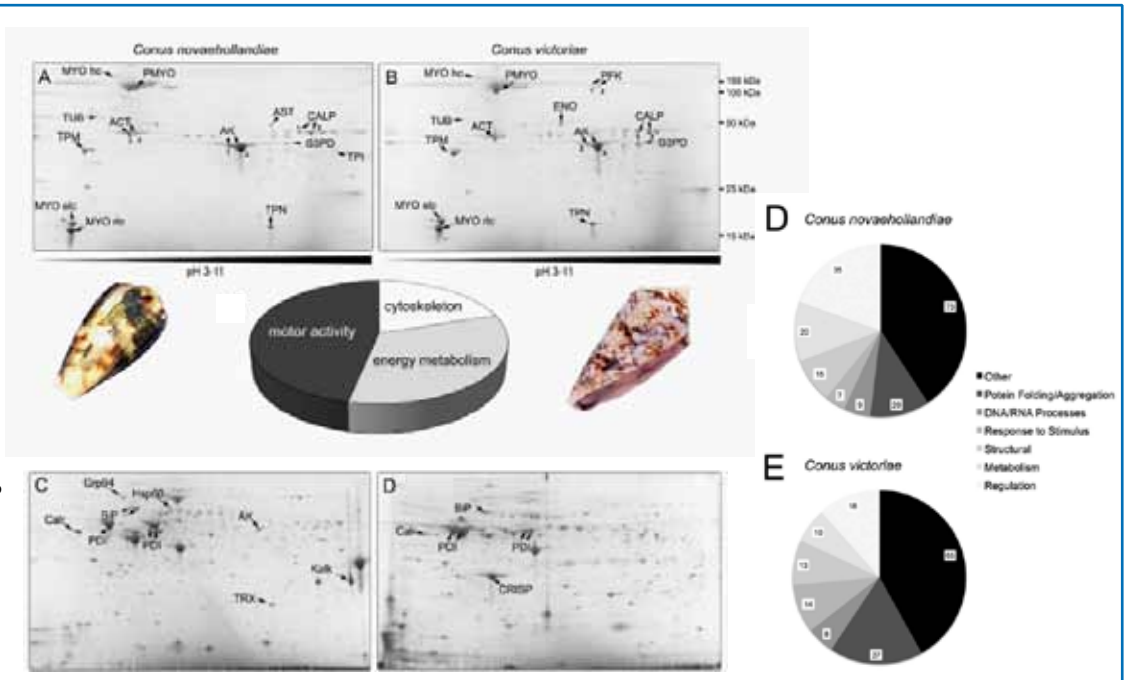

Fig. 3. 2D gel electrophoresis of proteins from *Conus novaehollandiae* (**A, C**) and Conus victoriae (**B, D**) venom bulbs and venom glands respectively. 200µg of total protein was loaded onto non-linear pH 3-11 IEF strips and separated on 8-16% SDS-PAGE gels. Gel spots were excised, digested and identified by LC-MS/MS. A subset of proteins is depicted. AK: arginine kinase, BiP: immunoglobulin heavy chain binding protein (Grp78), Calr: calreticulin, CRISP: cysteine rich secretory protein, Grp94: glucose-binding protein 94, Hsp60: heat shock protein 60, Kalk: kallikrein, PDI: protein disulfide isomerase, TRX: thioredoxin peroxidase, ACT: actin, AST: aspartate aminotransferase, BACT: beta actin, CALP: calponin, ENO: enolase, G3PD: glyceraldehyde 3-phosphate dehydrogenase, MYO: myosin, TPI: triosephosphate isomerase, TPN: troponin, TPM: tropomyosin, TUB: tubulin, PFK: phosphofructokinase, PMYO: paramyosin. A breakdown of the functional classes of proteins expressed is shown for each organ as pie charts.

different toxins utilising different isomers of enzymes such as PPI and PDI to achieve their optimal bioactive conformation. The collaboration of these enzymes by the formation of multi-enzyme complexes is also apparent. The molecular diversity of venom is maintained through post-transcriptional and post-translational control of toxin synthesis, folding and enzymatic modification.

Disulfide-rich peptides such as conotoxins are widely distributed throughout the animal and plant kingdom. Examples include antimicrobial peptides such as the defensins, proteinase inhibitors, hormones and neurotoxins from other venomous animals such as scorpions and snakes. Due to their complex structure, chemical synthesis of these disulfide-rich peptides is often associated with low folding yields and despite their importance, very little is known about their *in vivo* biosynthesis. Given the vast diversity of peptides generated by cone snails, conotoxins can be regarded as model disulfide-rich peptides. Thus, understanding enzyme and multi-enzyme assisted biosynthesis of conotoxins will provide a novel insight into the creation of chemical diversity in *Conus* species and shed light on fundamental mechanisms of disulfide-rich peptide biosynthesis.

References

1. Sharpe, I.A., Gehrmann, J., Loughnan, M.L., *et al.* (2001) *Nat. Neurosci.* **4**, 902-907
2. Davis, J., Jones, A., and Lewis, R.J. (2009) *Peptides* **30**, 1222-1227
3. Safavi-Hemami, H., Siero, W.A., Kuang, Z., *et al.* (2011) *J. Biol. Chem.* **286**, 22546-22557
4. Olivera, B.M. (2006) *J. Biol. Chem.* **281**, 31173-31177
5. Lopez-Vera, E., Walewska, A., Skalicky, J.J., Olivera, B.M., and Bulaj, G. (2008) *Biochemistry* **47**, 1741-1751
6. Loughnan, M.L., Nicke, A., Jones, A., *et al.* (2004) *J. Med. Chem.* **47**, 1234-1241
7. Buczek, O., Yoshikami, D., Watkins, M., *et al.* (2005) *FEBS J.* **272**, 4178-4188
8. Safavi-Hemami, H., Siero, W.A., Gorasia, D.G., *et al.* (2011) *J. Proteome Res.* **10**, 3904-3919
9. Safavi-Hemami, H., Young, N.D., Williamson, N.A., and Purcell, A.W. (2010) *J. Proteome Res.* **9**, 5610-5619
10. Garrett, J.E., Buczek, O., Watkins, M., Olivera, B.M., and Bulaj, G. (2005) *Biochem. Biophys. Res. Commun.* **328**, 362-367
11. Bulaj, G., and Olivera, B.M. (2008) *Antioxid. Redox Signal.* **10**, 1-15
12. Bulaj, G., Zhang, M.M., Green, B.R., *et al.* (2006) *Biochemistry* **45**, 7404-7414
13. DeLa Cruz, R., Whitby, F.G., Buczek, O., and Bulaj, G. (2003) *J. Peptide Res.* **61**, 202-212
14. Wang, Z.Q., Han, Y.H., Shao, X.X., Chi, C.W., and Guo, Z.Y. (2007) *FEBS J.* **274**, 4778-4787
15. Bulaj, G., Buczek, O., Goodsell, I., *et al.* (2003) *Proc. Natl. Acad. Sci. USA* **100**, 14562-14568
16. Gowd, K.H., Krishnan, K.S., and Balaram, P.I. (2007) *Mol. BioSyst.* **3**, 554–566
17. Goldberger, R.F., Epstein, C.J., and Anfinsen, C.B. (1963) *J. Biol. Chem.* **238**, 628-635
18. Pihlajaniemi, T., Helaakoski, T., Tasanen, K., *et al.* (1987) *EMBO J.* **6**, 643-649
19. Safavi-Hemami, H., Bulaj, G., Olivera, B.M., *et al.* (2010) *J. Biol. Chem.* **285**, 12735-12746
20. Meunier, L., Usherwood, Y.-K., Chung, K.T., and Hendershot, L.M. (2002) *Mol. Biol. Cell* **13**, 4456-4469