Spider vs Snake – discovery of novel snake venom protease inhibitors from spider venom

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Abstract; Serine Proteases are common in viper venoms such as the Trans-Pecos copperhead (*Agkistrodon contortrix pictigaster*). These venoms are procoagulant in their initial action through catalysis of Fibrinogen into insoluble fibrin. However, these snake venom serine proteases further catalyse the degradation of fibrin into fibrinopeptides that no longer form clots resulting in dysfibrinogenemia. In combination with other snake venom enzymes, such as SVMPs (Snake Venom MetalloProteases) which destroy the basement membranes and vascular integrity of snake bite victims, the dysfibrinogenemia results in major haemorrhage, characteristic of many viper bites. Theraphosid (Tarantula) spider venoms are well characterised with regards to ion channel inhibitors such as the Inhibitor Cysteine Knot (ICK) peptides, but inhibition of snake venom serine proteases is unreported. Our understanding of venom evolution is limited by the data we have so far. This study was to investigate if screening spider venoms for inhibitors of snake venom serine proteases was a valid hit finding strategy. To achieve this a Pierce® Fluorescent Protease Assay Kit (ThermoFisher) was used in 96 and 384 well format. The serine protease activity used was from the Trans-Pecos copperhead snake *Agkistrodon contortrix pictigaster* (*A.cpi*). Whole venom was assayed using a fluorescent casein substrate as part of a serine protease assay kit. Pre-incubation with a select range of theraphosid venoms quickly identified inhibition in an unexpected genus of spiders. Venom from the Brazilian Black Tarantula, *Grammostola pulchra*, was selected for further study as it was available in a fully fractionated library as part of the Calcium Channel Targeted Venom Discovery Array™ (T-VDACa^{2+}). Active hits from the fraction screen were analysed by mass spectrometry in order to characterise the molecular nature of the inhibitors. Initial experiments suggested two possible peptidic identities in different fractions. One was tentatively related to beta-theraphotoxins from tarantulas. The second was potentially related to a crotoxin subunit from phospholipase A2 (PLA2). Interestingly although PLA2 enzymes are widespread in snakes there are no previous reports of such proteins in spiders.
As far as we are aware this is the first demonstration of any spider venom having an inhibitory effect on any snake venom. The potential significance of these findings will be discussed in more detail in the poster. The discovery in this study has inspired an enzyme inhibitor Targeted-Venom Discovery Array™ (T-VDA™) as a new hit finding strategy.

Figure 1. Pictorial method

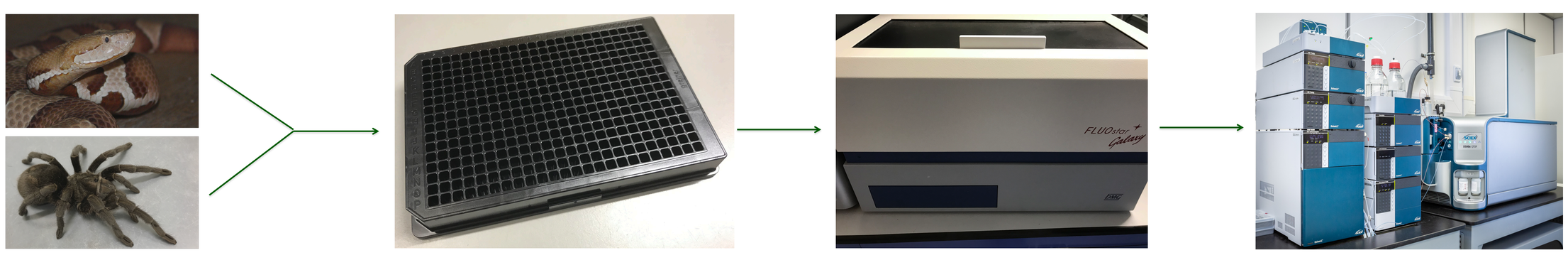


Figure 1 method, *Agkistrodon controtrix pictigaster* venom was characterised for its catalytic effect on fluorocasein Pierce® Fluorescent Protease Assay Kit (ThermoFisher) through determination of Vmax ad Km. *A.cpi* venom was diluted to 5ng/ul in tris-buffered saline, incubated with differing concentrations of spider venoms for 10 mins at room temperature, then the fluorescent substrate mix was added and incubated for different times, before reading fluorescence n the FLUOstar Galaxy (BMG Labtech). *Grammostola pulchra* venom was fractionated through cation exchange followed by reverse phase HPLC, fractions were standardised and lyophilised in 384 well plates. Venom fractions were screened as for crude venoms. Active hits were identified by intact mass and peptide mapping mass spectrometry .

Figure 2. Crude G.pul spider venom inhibition of *A.cpi* protease

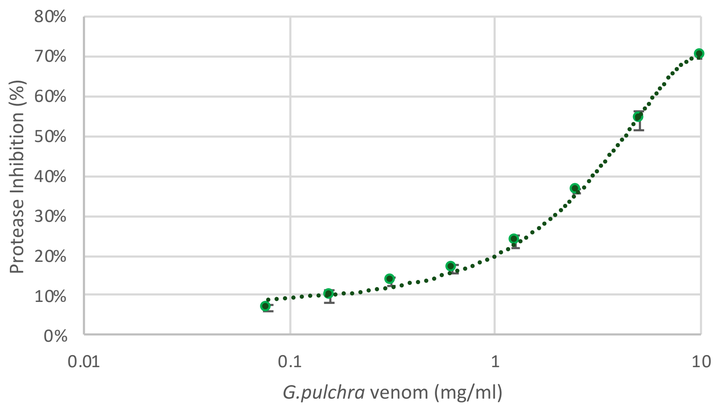
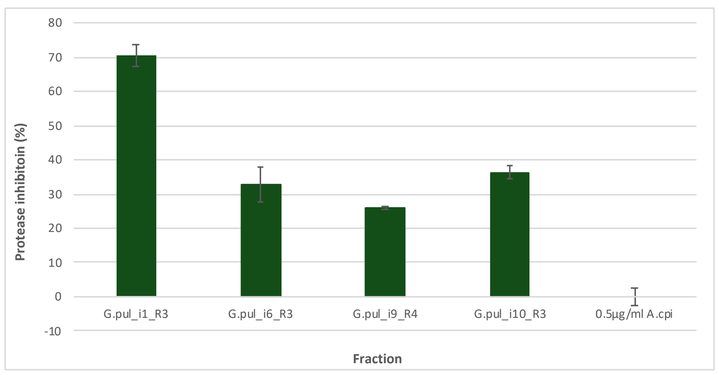


Figure 3. Individual fraction hits from *G.pul* venom

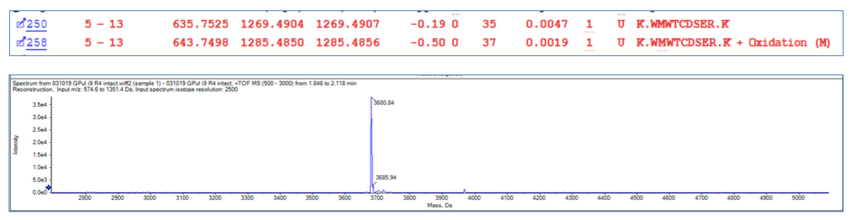


Conclusions

- Screening venom fractions is a viable hit finding strategy, even to inhibit other venoms
- Intact mass coupled with peptide mapping MS gives accurate data to distinguish novel peptides from those already deposited in databases
- Further work is required to determine the sequence of these novel protease inhibitor microproteins

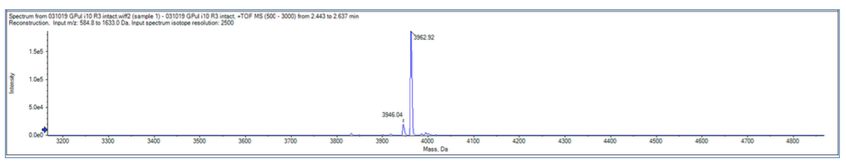
Figure 4. Mass Spectrometry data of active fractions

G.pul_i9_R4
Peptide mapping Analysis:One peptide matching TXPR2 – Beta/omega -theraphotoxin, other homologues identified. Sequence confirmed for peptide WMWTCDSER. Intact Mass Analysis: Main peak with a mass of 3680.84Da



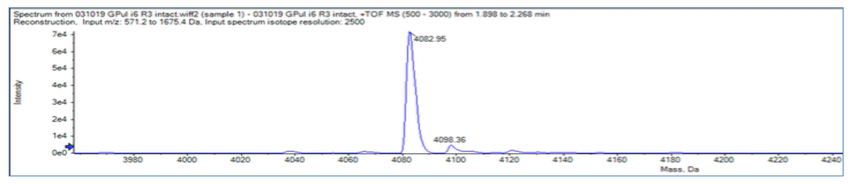
Query	Start – End	Observed	Mr(expt)	Mr(calc)	ppm	M	Score	Expect	Rank	U	Peptide
250	5 – 13	635.7525	1269.4904	1269.4907	-0.19	0	35	0.0047	1	U	K.WMWTCDSER.K
258	5 – 13	643.7498	1285.4850	1285.4856	-0.50	0	37	0.0019	1	U	K.WMWTCDSER.K + Oxidation (M)

G.pul_i10_R3
Peptide Mapping Analysis:
One peptide matching TX3_PARSR – Beta-theraphotoxin-Ps1a (Paraphysa scrofa) – sequence confirmed for peptide DCLGFLWK:Intact Mass Analysis:
Main peak with a mass of 3962.49Da.



Query	Start – End	Observed	Mr(expt)	Mr(calc)	ppm	M	Score	Expect	Rank	U	Peptide
207	1 – 8	519.7568	1037.4991	1037.5004	-1.33	0	45	0.0013	1	U	-.DCLGFLWK.C
208	1 – 8	519.7583	1037.5021	1037.5004	1.59	0	58	0.00028	1	U	-.DCLGFLWK.C

G.pul_i6_R3
Peptide Mapping Analysis:
One peptide matching PA1A – many homologues to phospholipase A2 homolog crotoxin acid subunit CA. Sequence confirmed for peptide AAAICFR. Intact mass Analysis:
Main peak identified at 4082.95Da



Query	Dupes	Observed	Mr(expt)	Mr(calc)	ppm	M	Score	Expect	Rank	U	Peptide
29		404.7109	807.4072	807.4061	1.38	0	38	0.026	1	U	K.AAAICFR.N

Peptide Mapping Analysis:
A 5ug sample was was reduced and alkylated followed by trypsin digestion. Then analysed using a Sciex X500B mass spectrometer coupled to a Sciex Exion LC. A 10 minute reverse phase gradient was used to analyse the samples. The flow from the column was passed into the Sciex X500B mass spectrometer collecting data in positive ion mode. MSMS data was collected using an Information Dependent Acquisition method where up to 10 MSMS were collected per scan. The X500B was calibrated with positive calibration mix, the error for this experiment was estimated at 1ppm. The data was searched against Swissprot using Mascot.

Intact Mass Analysis:
The 5ug lyophilised sample was reconstituted and loaded onto the Sciex Exion LC with a 5 minute reverse phase gradient on a Phenomenex Jupiter 5um, C4, 300A 50x2.1mm column. The flow from the column was passed into the Sciex X500B mass spectrometer collecting data in positive ion mode. The X500B was calibrated with positive calibration mix, the error for this experiment was estimated at 0.1Da. The resultant TIC was deconvoluted using BioToolKit software.

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