Liquid chromatographic nanofractionation with parallel mass spectrometric detection for the screening of plasmin inhibitors and (metallo)proteinases in snake venoms

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S1.1 Plasmin bioassay development and optimization

The plasmin bioassay is a fluorescence-based bioassay in which conversion of the fluorogenic substrate H-D-Val-Leu-Lys-AMC by plasmin to its fluorescent product 7-amino-4-methylcoumarin is measured. The bioassay developed for this study is based on the protocol published by Tervo et al.[1]

The optimization procedure included selection of the concentration and pH of the buffer, the concentration of plasmin and of H-D-Val-Leu-Lys-AMC, and optimization of the well volume. A Tris-HCl buffer was chosen and tested at 50 and 100 mM, and at pH 7.0, 7.5, 7.8 and 8.0. For these experiments, the enzyme and the substrate were diluted in the respective buffers to final concentrations of 100 ng/ml and 5 µM, respectively. The measurements were replicated six times using black, 384-well format, microtiter well plates. The fluorescence was measured kinetically with a VarioSkan LUX microplate reader set to an excitation and emission wavelength of 380 and 460 nm, respectively, with a bandwidth of 12 nm. The measurements were performed at 37 °C. The 100 mM buffer (pH 7.5) showed the best performance (i.e. showing the smallest signal variation between replicates and the highest fluorescence signal intensity) (Figures S3a and S3b for 100 mM and 50 mM, respectively).

Using the optimized buffer, ten H-D-Val-Leu-Lys-AMC concentrations between 0 and 50 µM were tested at an enzyme concentrations of 100 ng/ml. Substrate at each concentration was mixed with plasmin, and the product formation was measured in time. For each well, an enzymatic reaction curve was generated and all curves were then used to calculate K_M value (31 µM) using the Michaelis-Menten kinetic model using Prism v.5 software (Figure S3c). At 5.0 µM substrate a stable baseline with a satisfying S/N ratio was obtained. Considering the high price of the substrate, 5 µM substrate was chosen for the bioassay.

For selection of the optimal assay volume, the bioassay mixture was tested at 25, 50 and 75 µL volumes. For each tested volume, bioassay mixture was pipetted into 300 wells of a black 384-well microtiter plate using a Multidrop 384 reagent dispenser. The enzymatic activity was measured over 10 cycles. The slope for each well over these 10 cycles was calculated and plotted against the well number. The readout of the well plate was performed in a serpentine way (from well 1A to 1K, and then from 2K to 2B, etc. covering the whole 384 well plate). This readout fashion will mimic the readout of a nanofractionated plate, also performed in a serpentine way. The data was plotted as if it was a “blank” nanofractionated plate for which the signal should be the same in all wells. This way discrepancies can now
nicely be observed since edge effects are observed as regular zigzag patterns in the plotted data. From these results the optimal mixture volume (i.e. the data showing the least differences in highest and lowest signals, and the smallest zigzag pattern) was determined to be 50 µL (Figure S3d).

The fully optimized plasmin bioassay mixture (50 µL) comprised 100 ng/ml plasmin and 5 µM substrate in 100 mM Tris-HCl buffer (pH 7.5) with 0.1% BSA.

S1.2 NanoLC-MS/MS analysis of tryptic digests

The nanoLC system consisted of a NCS-3500RS Nano/Cap System with a NCP-3200RS Nano/Cap Pump and WPS-3000(RS) Autosampler UltiMate 3000 Series from Thermo Scientific. Prior to separation, samples were pre-concentrated on a C18 trapping column (Acclaim™ PepMap™ 100 C18 LC Columns, 5µm, 5mm length, 0.3mm I.D. from Thermo Fisher Scientific) connected to the analytical column (Acclaim™ PepMap™ 100 C18 LC Columns, 2µm, 150mm length, 0.75mm I.D. from Thermo Fisher Scientific) via a 6-port switch valve. Both columns were kept at 45 °C. Analyses started by injecting 1 µL of sample onto the trapping column via a 20 µL injection loop (the injection mode used was µL-pickup, in which sample is aspirated with help of a transport liquid, which was pure water) at a flow rate of 10 µL/min using the sample loading pump. At this stage, the trapping column was not in line with the nanoflow solvent (NS) pumps. After 4 min the 6-port valve was switched to the position in which the pre-column was in line with the NS pumps enabling the trapped peptides and proteins to be eluted onto the analytical column. The flow rate of the NC pump was maintained at 0.5 µL/min throughout the analysis. Separation was performed using binary gradient elution with solvent A comprising water with 0.1% FA (v/v) and solvent B comprising 80% ACN, 20% water and 0.1% FA (v/v/v). The following gradient was used for separation (after trapping 4 min): from 4-10 min an isocratic flow at 1% B, which increased to 20% B in 10-15 min. From 15-45 min, the percentage B increased to 45% and next within 1 min it increased to 85% B, where it remained for 5 min. Then in 0.5 min it returned to initial conditions. Column equilibration time was set at 8.5 min. At 59 min, the 6-port valve was switched back to the position in which the trapping column was in line with the loading pump delivery system for the next 1 min. The nanoLC system was controlled by Chromeleon version 7.2 SR4 software. A Maxis HD quadrupole time-of-flight MS (Bruker Daltonics, Bremen, Germany) was operated in positive electrospray ionization (ESI) mode with a capillary voltage set at 1.3-1.4 kV, a dry gas flow rate of 3 l/min and dry gas temperature set
at 150 °C. Analyses were performed using auto MS/MS scan mode. Full mass spectra were acquired (m/z 500-2000), from which the three most intense ions per scan were fragmented using collision induced dissociation (CID) in stepping mode. The parameters used for fragmentation were: collision energy, 80-100%; timing, 50%. Parameters used for the basic stepping can be found in Table S1. Mass accuracy of 1-2 ppm was ensured by calibrating the system with an ESI-L Low concentration Tuning Mix (Agilent, Santa Clara, California, USA).

S2.1 Detailed plasmin bioassay results of the six bioassay variants

_Cb_ and _Dr_ were investigated for the presence of proteinases and their possible calcium and/or zinc dependency. The two venoms (4 mg/ml each) were nanofractionated and next exposed to six different variants of the plasmin bioassay, which are described in detail in the main manuscript of this study (Section “Profiling plasmin inhibition and proteolytic activity in _Cb_ and _Dr_ venoms”).

Starting with the first variant, in which no plasmin was used, the bioactivity chromatograms are shown in Figure 3b for _Cb_ and _Dr_. For clarity, first the results of _Cb_ venom and then these of _Dr_ venom will be discussed. Considering that the bioactive peak in _Cb_ venom did not disappear when running the bioassay without plasmin, most probably other proteases present in the venom are directly responsible for conversion of the substrate to its fluorescent product. Venom derived from _Cb_ is known to be rich of proteolytic enzymes that show fibrino(gen)lytic properties. An example includes basilase, which is a metalloprotease containing zinc and calcium ions (1 and 2 mol per mol of protein, respectively), suggesting that its activity is dependent on both metal ions [2]. Assessment of the type of protease identified in the bioactivity chromatogram, i.e. whether it belongs to a family of metalloproteases or serine protease, was performed by exposing nanofractionated snake venom to the second and third variant of the bioassay mixture (i.e. without plasmin and with the addition of zinc (0.2 mM) or calcium (1 mM) ions, respectively). An increase of a bioactivity peak after addition of any of the two ions suggests dependence of the bioactive protein on the particular metal ion added. Zinc and calcium ions are known constituents of snake venom proteases, which are vital for the catalytic activity of metalloproteinases and also stabilize their structural conformation [3]. The results of the bioassays were compared to the ones in which only the substrate was used (Figure 3a). Figure 3d shows a threefold increase when the venom was exposed to a bioassay mixture enriched with calcium ions. The
addition of zinc ions, however, showed no significant changes in intensity of the bioactivity peak (Figure 3c).

Variation between plates can be expected as introduced by the chromatographic analyses, the time between placing venom in the cooled autosampler and actual analysis, the time between nanofractionation and vacuum centrifugation, the duration of vacuum centrifugation, as well as small changes in activity of the bioassay constituents during bioassay preparation. In order to assure that the variations observed in the bioassay are not a result of the variation between different plates/bioassays tested, additional experiments confirming the dependence of the bioactive compounds on the metal ions were performed. In these experiments two bioassay mixtures, one enriched with calcium ions and the other with zinc ions, were pipetted into alternating wells of one microtiter well plate containing nanofractionated venom. Here it has to be noted that the nanofractionation is done in serpentine fashion with filling the wells of columns of a well plate from left to right. Pipetting alternating wells with calcium or zinc solutions is done perpendicular to the nanofractionation pattern (i.e. alternating rows of a well plate are filled). This experiment confirmed a positive influence of calcium ions on the eluted bioactive proteins, as a twofold higher intensity of the bioactive peak was observed.

Finally, the presence of metalloproteases was further confirmed by an experiment in which the metal ion chelator 1,10-phenanthroline (5 mM) or EDTA (50 mM) was added to the bioassay mixture. Figure 3f shows a bioactivity chromatogram of Cb venom after 1,10-phenanthroline addition in which the bioactivity peak intensity did not change compared to the bioactivity chromatogram obtained from the experiment without the zinc chelator (Figure 3c). EDTA, which is known to chelate divalent ions including calcium and zinc, was observed to decrease the intensity of the activation peak by twofold (Figure 3e). The decrease of the peak intensity in this experiment indicated that proteases present in the Cb venom probably are calcium ion dependent. To reassure that the lower intensity of the bioactivity peak was indeed influenced by the addition of EDTA, and not by earlier mentioned variations observed between measurements, also here bioassay mixtures with and without EDTA were pipetted into alternating wells onto one microtiter well plate containing nanofractionated Cb venom. The results of this experiment (Figure S4b) confirmed that by removing calcium ions from the sample, the bioactivity decreased supporting the conclusion that indeed the positive peak observed was at least partly resulting from a calcium dependent proteolytic enzyme. Another explanation to the observed results could be that the positive peak contains two or more proteases, in which only one is calcium dependent. The removal of calcium ions would
cause a substantial decrease in the activity peak of the calcium dependent proteases, however not for other non-calcium dependent ones.

In case of *Dr* venom, the use of bioassay mixture without plasmin addition (first variant of the bioassay mixture) resulted in disappearance of the negative peaks, as plasmin was not present in the bioassay mixture, and no presence of activation peaks was observed either (Figure 3b). The addition of zinc ions (variant 2) and addition of calcium ions (variant 3, Figure 3d) to the bioassay mixture not containing plasmin showed no bioactivity. A further experiment in which EDTA (variant 5) was added to the bioassay mixture containing only the substrate, showed no bioactivity (Figure 3e). Addition of 1,10-phenanthroline to the mixture containing the enzyme and substrate revealed two additional negative peaks between 14 and 18 min in the bioactivity chromatogram (Figure 3f). These negative peaks might represent additional plasmin inhibitors, which due to a possible co-elution with metal dependent proteases that were not detected possibly due to their low concentration or low stability, could not be identified after exposing the nanofractionated venom to the normal variant of the bioassay (i.e. only containing plasmin and the substrate). Therefore, the two negative peaks most probably are plasmin inhibitors.

References:

1. Tervo T, Honkanen N, Setten G-B van, Virtanen T, Tarkkanen A, Harkonen M (1994) A rapid fluorometric assay for tear fluid plasmin activity. Cornea 13:148–155.
2. Datta G, Dong A, Witt J, Tu AT (1995) Biochemical Characterization of Basilase, a Fibrinolytic Enzyme from Crotalus basiliscus basiliscus. Arch Biochem Biophys 317:365–373.
3. Takeya H, Nishida S, Nishino N, Makinose Y, Omori-Satoh T, Nikai T, Sugihara H, Iwanaga S (1993) Primary structures of platelet aggregation inhibitors (disintegrins) autoproteolytically released from snake venom hemorrhagic metalloproteinases and new fluorogenic peptide substrates for these enzymes. J Biochem 113:473–483.
Fig. S1 Nanofractionation system with parallel MS data acquisition. After nanofractionation and vacuum centrifuge drying of the 384-well plate, a fluorescence plasmin activity assay is performed. (I) Chromatographic separation of snake venom; (II) Split (10% of the column effluent) to MS analysis; (III) UV detection followed by (IV) high resolution (6 s) nanofractionation of 90% of the column effluent; (IV’) MS analysis; (V) Evaporation of solvent; (VI) Bioassay mixture addition: a) addition of the full bioassay mixture containing the enzyme and the substrate, b) addition of the bioassay mixture enriched with zinc ions (yellow) or calcium ions (red) in alternative way; c) addition of the full bioassay mixture to every second well of the well plate; (VII) Fluorescence readout; (VIII) Selection of wells with bioactive proteins for tryptic digestion; (IX) Analysis of tryptic digests using nanoLC-MS/MS; (X) Identification of bioactive proteins using Mascot database search.
Fig. S2 Time alignment of the nanofractionated bioassay and the MS data using leupeptin for (A) RPLC analysis, and (B) HILIC analysis. I, bioactivity chromatograms; II, extracted ion chromatograms. Leupeptin [M+H]$^+$ is 427.3025 Da.
Fig. S3 Bioassay optimization: (a) 50 mM TRIS-HCl buffer at various pH; and (b) 100 mM TRIS-HCl buffer at various pH; (c) Determination of $K_M$ value at 100 ng/ml plasmin and ten substrate concentrations ranging between 0 and 50 µM. (d) Bioassay mixture volume optimization using 100 ng/ml plasmin and 5 µM substrate in 0.1 M TRIS-HCl buffer with 0.1 % BSA
Fig. S4 Bioassay chromatograms of *Cb* venom nanofractionated on a single plate and exposed to two different bioassay mixtures using the alternating well method. (a) Plasmin bioassay with alternating zinc/calcium enrichment; (b) Plasmin bioassay with alternating EDTA/no EDTA addition.
Table S1 Isolation and fragmentation parameters used for nanoLC-MS/MS analysis of the digested bioactive proteins and peptides found in *Cb* and *Dr*.

| Type  | Mass | Width | Collision | Charge state |
|-------|------|-------|-----------|--------------|
| Base  | 500  | 8     | 35        | 1            |
| Base  | 500  | 8     | 30        | 2            |
| Base  | 500  | 8     | 25        | 3            |
| Base  | 500  | 8     | 20        | 4            |
| Base  | 1000 | 10    | 50        | 1            |
| Base  | 1000 | 10    | 40        | 2            |
| Base  | 1000 | 10    | 35        | 3            |
| Base  | 1000 | 10    | 30        | 4            |
| Base  | 2000 | 15    | 70        | 1            |
| Base  | 2000 | 15    | 50        | 2            |
| Base  | 2000 | 15    | 45        | 3            |
| Base  | 2000 | 15    | 40        | 4            |
Table S2 Mascot search results for *Cb* bioactives. The information in the table includes: The accession number, protein score, protein name, protein % coverage, number of matches, Mr Obs (observed molecular mass), Mr Exp (expected molecular mass, Mr Calc (calculated molecular mass), peptide score, and Expect (expectation value).

| Accession number | Protein score | Protein name | % Coverage | No of matches | Number of distinct sequences | Mr Obs. | Mr Exp. | Mr Calc. | Peptide Score | Expect. | Peptide sequence |
|------------------|---------------|--------------|------------|--------------|-----------------------------|---------|---------|----------|---------------|---------|-----------------|
| VSP_CRODD        | 163           | Snake venom serine protease OS=Crotalus durissus durissus PE=2 SV=1 | 17         | 7            | 3                           | 559.7972 | 1117.5799| 1117.5801| 70            | 0.00098 | R.TLCAGILEGGK.G |
|                  |               |              |            |              |                             |         |         |          |               |         |                 |
|                  |               |              |            |              |                             | 1249.5829 | 2497.1512| 2497.1472| 63            | 7.30E-05| K.VFDYTEWIQSII AGNTDATCP.- |
|                  |               |              |            |              |                             | 504.9026 | 1511.6858| 1511.6787| 44            | 4.00E-03| L.VIGGDECNINE HR.S |
| VSP1_CROAT       | 129           | Snake venom serine protease catroxase-1 OS=Crotalus atrox PE=1 SV=1 | 24         | 6            | 4                           | 559.7972 | 1117.5799| 1117.5801| 70            | 0.00098 | R.TLCAGILEGGK.D |
|                  |               |              |            |              |                             | 698.348  | 1394.6814| 1394.663  | 61            | 0.004    | R.AAYPEYGLPAT SR.T |
|                  |               |              |            |              |                             | 715.358  | 1428.7014| 1428.7031 | 73            | 0.00016  | L.SLPSSPSVGSV CR.I |
|                  |               |              |            |              |                             | 960.792  | 2879.3543| 2879.3589 | 48            | 0.00015  | K.VFDHLDWIQSII AGNTDATCPFVN F.- |
| VSP2_CROAT       | 82            | Snake venom serine protease catroxase-2 OS=Crotalus atrox PE=1 SV=1 | 12         | 2            | 2                           | 500.2303 | 1497.6691| 1497.663  | 55            | 0.0002   | L.VVGDECNINE HR.S |
|                  |               |              |            |              |                             | 714.7009 | 2141.0808| 2141.0463 | 61            | 0.00027  | K.LLDDAVCQPPY PELPATS.R.T |
| VSPY_MACLB       | 71            | Chymotrypsin-like protease VLCLTP OS=Macrovipera lebetine PE=1 SV=1 | 13         | 4            | 2                           | 500.2303 | 1497.6691| 1497.663  | 55            | 0.0002   | L.VVGDECNINE HR.S |
|                  |               |              |            |              |                             | 824.0618 | 2469.1637| 2471.1679 | 39            | 0.0077   | K.VFDYSDWIQSII AGNTATCPL.- |
| Code         | Description                                                                                     | OS          | PE | SV | MW (Da) | pI | EC | Enzymatic Activities                                                                 |
|--------------|------------------------------------------------------------------------------------------------|-------------|----|-----|---------|----|----|-------------------------------------------------------------------------------------|
| WM1AD_CROAT  | Snake venom metalloproteinase atrolysin-D                                                     | Protobothrops jerdonii | 1  | 3   | 536.6153 | 61 | 0.0005    | R.VHEIVNFINGFYR.S                                                                   |
| VSP2_PROJR   | Snake venom serine protease 3 OS=Protobothrops jerdonii PE=2 SV=1                              | 8           | 5  | 1   | 1242.0646 | 65 | 1.40E-05 | N.VFNYTDWIQSIIAGNTDATCPP.-                                                            |
| PA2AF_CROVV  | Acidic phospholipase A2 Cvv-Edf OS=Crotalus viridis viridis PE=1 SV=1                           | 26          | 4  | 3   | 722.3081  | 53 | 0.0002    | R.SGLFWYGAYG CY.C                                                                     |
|              |                                                                                               |             |    |     |         |    |    |                                                                                     |
|              |                                                                                               | 565.2405    | 1692.6996 | 1691.7131 | 53 | 0.0045    | K.NGEIICEDDPCKK.Q                                                                     |
|              |                                                                                               | 502.5192    | 1504.5357 | 1504.5356 | 35 | 0.0044    | R.CCFVHDCCYG K.A                                                                     |
| VM2I_CROBA   | Disintegrin basilicin OS=Crotalus basiliscus PE=1 SV=1                                          | 23          | 1  | 1   | 684.2932  | 77 | 1.90E-06 | K.LRPGAQCAEGLCCDQCR.F                                                                 |
|              |                                                                                               |             |    |     |         |    |    |                                                                                     |
|              |                                                                                               | 698.346     | 1394.6775 | 1394.683  | 73 | 0.00061   | R.AAYPEYGLPAT SR.T                                                                   |
|              | VSP_CRODD                                                                                     | 233         | 13  | 8   | 1249.5779 | 84 | 1.70E-07 | K.VFDYTEWIQSIIAGNTDATCPP.-                                                            |
|              |                                                                                               |             |    |     |         |    |    |                                                                                     |
|              |                                                                                               | 504.8994    | 1511.6763 | 1511.6787 | 47 | 0.0025    | L.VIGGDENCINE HRS                                                                    |
|              | VSP1_CROAT                                                                                     | 99          | 10  | 2   | 715.3554  | 76 | 6.20E-05 | L.SLPSSPPSVGSV CR.I                                                                   |
|              |                                                                                               |             |    |     |         |    |    |                                                                                     |
|              |                                                                                               | 698.346     | 1394.6775 | 1394.683  | 73 | 0.00061   | R.AAYPEYGLPAT SR.T                                                                   |
|              | VSP2_CROAT                                                                                     | 91          | 17  | 3   | 739.8746  | 45 | 0.016     | K.LDSPVSNSEHIA PL.S                                                                  |
|              |                                                                                               |             |    |     |         |    |    |                                                                                     |
|              |                                                                                               | 500.2308    | 1497.6707 | 1497.663  | 40 | 0.0036    | L.VVGGDECNINE HRS                                                                    |
|              |                                                                                               | 714.6984    | 2141.0735 | 2141.0463 | 74 | 6.90E-06 | K.LLDDAVCPQPPY PELPATS R.T                                                            |
|              | VSP3_PROJR                                                                                     | 100         | 8   | 4   | 1242.057  | 52 | 0.00014  | N.VFNYTDWIQSII AGNTDATCPP.-                                                            |
|              |                                                                                               |             |    |     |         |    |    |                                                                                     |
| PA2AF_CROVV | 59 | Acidic phospholipase A2 Cvv-E6f OS=Crotalus viridis viridis PE=1 SV=1 | 18 | 2 | 2 | 502.5176 | 1504.5309 | 1504.5356 | 39 | 0.0028 | R.CCFVHDCCYG K.A |
|------------|----|-------------------------------------------------|----|---|---|---------|---------|---------|---|-------|----------------|
|            |    |                                                 |    |   |   | 564.9099 | 1691.7078 | 1691.7131 | 51 | 0.0013 | K.NGEIICEDDDPC KK.Q |
**Table S3** Mascot search results for *Dr* bioactives. The information in the table includes: The accession number, protein score, protein name, protein % coverage, number of matches, Mr Obs (observed molecular mass), Mr Exp (expected molecular mass, Mr Calc (calculated molecular mass), peptide score, and Expect (expectation value).

**Daboia russellii HILIC**

| Accession number | Protein score | Protein name | % Coverage | No of matches | Mr Obs. | Mr Exp. | Mr calc. | Score | Expect. | Peptide sequence |
|------------------|---------------|--------------|------------|---------------|---------|---------|----------|-------|---------|------------------|
| PA2B8_DABRR      | 2173          | Basic phospholipase A2 VRV-PL-VIIa OS=Daboia russellii PE=1 SV=1 | 80 | 63 | 14 | 471.7264 | 941.4383 | 941.4317 |        | 0.036 | L.YPDFLCK.G |
|                  |               |              |            |               |         |         |          |       |         | 484.2488 | 966.4831 | 966.4377 | 69 | 0.05 | R.QNLNTYSK.K |
|                  |               |              |            |               |         |         |          |       |         | 495.2599 | 988.5053 | 988.5012 | 68 | 0.00025 | R.VNGAIVCEK.G |
|                  |               |              |            |               |         |         |          |       |         | 675.3211 | 1348.6277 | 1348.6719 | 47 | 0.00018 | K.YMLYPFLCK.G |
|                  |               |              |            |               |         |         |          |       |         | 683.3174 | 1364.6203 | 1364.6145 | 47 | 0.029 | K.YMLYPFLCK.G + Oxidation (M) |
|                  |               |              |            |               |         |         |          |       |         | 691.2724 | 1380.5302 | 1380.5227 | 75 | 6.80E-06 | Y.SSYGCYCGWG G.K.G |
|                  |               |              |            |               |         |         |          |       |         | 729.3143 | 1456.614 | 1456.6149 | 41 | 9.10E-03 | R.ICECDKAAAICF R |
|                  |               |              |            |               |         |         |          |       |         | 538.5818 | 1612.7236 | 1612.716 | 73 | 5.30E-06 | R.ICECDKAAAICF R.Q |
|                  |               |              |            |               |         |         |          |       |         | 892.3818 | 1782.7491 | 1782.7382 | 74 | 2.60E-03 | K.LAIPSYSSYGCY CGW.G |
|                  |               |              |            |               |         |         |          |       |         | 897.4021 | 1792.7897 | 1792.8196 | 109 | 9.20E-09 | R.VNGAIVCEKGT SCENR.I |
|                  |               |              |            |               |         |         |          |       |         | 598.6039 | 1792.7899 | 1792.8196 | 77 | 8.60E-07 | R.VNGAIVCEKGT SCENR.I |
|                  |               |              |            |               |         |         |          |       |         | 646.3085 | 1935.9038 | 1935.8933 | 46 | 1.90E-03 | K.YMLYPFLCK GELK.C. |
|                  |               |              |            |               |         |         |          |       |         | 650.6381 | 1948.8923 | 1948.9207 | 83 | 3.10E-07 | K.RVNGAIVCEKGT TSCENR.I |
|                  |               |              |            |               |         |         |          |       |         | 675.9692 | 2024.8857 | 2024.8761 | 82 | 3.00E-07 | K.LAIPSYSSYGCY CGWGGK.G |
|                  |               |              |            |               |         |         |          |       |         | 1013.4503 | 2024.8861 | 2024.8761 | 112 | 8.90E-10 | K.LAIPSYSSYGCY CGWGGK.G |
| Accession   | Year | Description                                      | Start | Length | Mass 1 | Mass 2 | Mass 3 | Charge | Mass 4 | PDB    |
|-------------|------|--------------------------------------------------|-------|--------|--------|--------|--------|--------|--------|--------|
| PA2B5_DABRR | 1935 | Basic phospholipase A2 VRV-PL-V OS=Daboia russelii PE=1 SV=1 | 71    | 16     | 471.7264 | 941.4383 | 941.4 | 0.036  | L.YPDFLCK.G |
|             |      |                                                  |       |        | 484.2488 | 966.4831 | 966.5 | 0.05   | T.KNLNTYSK.I |
|             |      |                                                  |       |        | 495.2599 | 988.5053 | 988.4 | 0.00025 | R.VNGAIVCEQ.G |
|             |      |                                                  |       |        | 675.3211 | 1348.6277 | 1348.6 | 0.00018 | I.YMLYPDFLCK.G |
|             |      |                                                  |       |        | 683.3174 | 1364.6203 | 1364.6 | 0.029  | I.YMLYPDFLCK.G |
|             |      |                                                  |       |        | 691.2724 | 1380.5302 | 1380.5 | 6.80E-06 | Y.SSYGCYCGWG |
|             |      |                                                  |       |        | 713.8253 | 1425.636 | 1425.6 | 0.0024 | K.LAVPFSYSGYCY.C |
|             |      |                                                  |       |        | 729.3143 | 1456.614 | 1456.6 | 0.0091 | R.ICECDKAAAICF.T |
|             |      |                                                  |       |        | 731.8638 | 1461.7131 | 1461.7 | 0.0013 | K.IYMLYPDFLCK.G |
|             |      |                                                  |       |        | 739.8609 | 1477.7072 | 1477.7 | 0.00015 | K.IYMLYPDFLCK.G |
|             |      |                                                  |       |        | 849.4331 | 1696.8516 | 1696.8 | 3.60E-09 | -SLLEFGMMILEETGK.L |
|             |      |                                                  |       |        | 857.4293 | 1712.844 | 1712.8 | 3.90E-08 | -SLLEFGMMILEETGK.L |
|             |      |                                                  |       |        | 857.4301 | 1712.8456 | 1712.8 | 1.40E-08 | -SLLEFGMMILEETGK.L |
|             |      |                                                  |       |        | 865.4265 | 1728.8385 | 1728.8 | 1.20E-05 | -SLLEFGMMILEETGK.L |
|             |      |                                                  |       |        | 897.4021 | 1792.7897 | 1792.8 | 9.20E-09 | R.VNGAIVCEQGT |

Notes: (M) indicates oxidation.
| Protein   | Accession | Description                                                                 | Match Count | Match | Score |
|-----------|-----------|------------------------------------------------------------------------------|-------------|-------|-------|
| PA2B_VIPRE| 1463      | Basic phospholipase A2 vurtoxin OS=Vipera renardi PE=1 SV=1                  | 29          | 35    | 4     |
|           |           |                                                                              | 477.7264    | 941.4383 | 941.4383 | 47    | 0.036 | Y.YPDFLCK.K |

849.4331 | 1696.8516 | 1696.8416 | 117 | 3.60E-09 | G.SLLEFGMMILEETGK.N |
| 857.4293 | 1712.844 | 1712.8365 | 104 | 3.90E-08 | G.SLLEFGMMILEETGK.N + Oxidation (M) |
| 857.4301 | 1712.8456 | 1712.8365 | 110 | 1.40E-08 | G.SLLEFGMMILEETGK.N + Oxidation (M) |
| 865.4265 | 1728.8385 | 1728.8314 | 73 | 1.20E-05 | G.SLLEFGMMILEETGK.N + 2 Oxidation (M) |
| 877.9431 | 1753.8716 | 1753.863 | 95 | 1.70E-07 | E.GSLLEFGMMILEETGK.N |
| 772.6319 | 2314.8738 | 2314.8687 | 73 | 1.10E-05 | R.CCFVHDCCYGNLPCNPK.I |

| Protein   | Accession | Description                                                                 | Match Count | Match | Score |
|-----------|-----------|------------------------------------------------------------------------------|-------------|-------|-------|
| VKT3_DABRR| 65        | Kunitz-type serine protease inhibitor 3 OS=Daboia russelli PE=2 SV=1         | 20          | 1     | 1     |
|           |           |                                                                              | 651.9631    | 1952.8674 | 1952.8588 | 65    | 0.0063 | K.EFIYGCCHGNA NNFPTR.D |

| Protein   | Accession | Description                                                                 | Match Count | Match | Score |
|-----------|-----------|------------------------------------------------------------------------------|-------------|-------|-------|
| PA2AL_TRIST| 63       | Acidic phospholipase A2 CTs-A3 OS=Trimeresurus stejnegeri PE=1 SV=1         | 11          | 1     | 1     |
|           |           |                                                                              | 557.5885    | 1669.7437 | 1669.7375 | 63    | 0.00017 | K.AVCECKAAAAI CFR.D |
| Daboia russelii RP C18 |
|------------------------|
| PA2B_VIPRE 493 Basic phospholipase A2 | vurtoxin OS=Vipera renardi PE=1 SV=1 |
| 11 11 2 | 849.4249 1696.835 1696.8416 94 1.60E-07 |
| | G.SLLEFGMMILE ETGK.N |
| 877.9387 1753.862 1753.863 96 2.40E-07 |
| | E.GSLLEFGMMIL ETGK.N |
| VKTC6_DABSI 343 Kunitz-type serine protease inhibitor C6 OS=Daboia siamensis PE=2 SV=1 |
| 43 8 5 | 677.2835 1352.552 1352.5528 66 0.00029 |
| | Y.GGC GGNNANNF ETR.D |
| 798.3331 1594.651 1594.651 78 4.00E-07 |
| | R.FYYNPASNQCQ GF.T |
| 367.1814 1464.696 1464.7004 32 0.03 |
| | R.HTCGASGNVGP RPR.I |
| 809.3397 1616.664 1616.6638 140 3.30E-12 |
| | F.TYG CCGGN AN NFETR.D |
| 1065.442 3193.304 3193.3043 128 2.20E-12 |
| | R.FYYNPASNQCQ GFTYGGCGGNAN NFETR.D |
| VKT4_DABRR 125 Kunitz-type serine protease inhibitor 4 OS=Daboia russelii PE=2 SV=1 |
| 33 3 3 | 677.2835 1352.552 1352.5528 66 0.00029 |
| | Y.GGC GGNNANNF ETR.D |
| 798.3331 1594.651 1594.651 78 4.00E-07 |
| | R.FYYNPASNQCQ GF.T |
| 814.8215 1627.628 1628.7001 45 0.00079 |
| | F.TYG CCGGN AN NFETR.D |
| VKT3_DABRR 58 Kunitz-type serine protease inhibitor 3 OS=Daboia russelii PE=2 SV=1 |
| 36 4 2 | 855.3362 1708.65 1708.6572 33 0.02 |
| | K.FCYLPADPGE CMAY.I + Oxidation (M) |
