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Application News

No. MO379

AXIMA Performance MALDI TOF-TOF

Disulfide bond rearrangement study using MALDI-TOF TOF mass spectrometry with high and low-energy fragmentation.

Ammodytoxins (Atxs) are a group of potent neurotoxins present in the venom of the long-nosed viper (Vipera ammodytes ammodytes). In particular, Atxs (A, B and C) exhibit presynaptic toxicity interfering with the release of acetylcholine from synapses which induces cell damage, apoptosis and ultimately death. Atxs exhibit extensive sequence homology sharing 117 residues in a 122 amino acid sequence. Within the Atxs, there are 7 biologically relevant disulfide bridges between the 14 cysteine residues present: these are highlighted in the schematic in figure 1

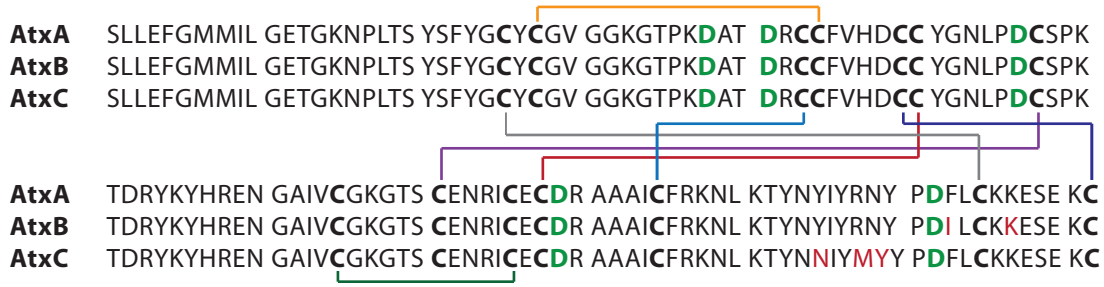

Figure 1: Amino acid sequence and disulfide bridge map of ammodytoxins A, B and C. Bold red denotes amino acid disparities and bold green denote formic acid cleavage sites.

Disulfide bridge rearrangement:

The Atxs sample was cleaved using formic acid for sequence characterisation. Once cleaved, the samples were purified using ZipTip™ with both aqueous and organic fraction elution. The peptide mass fingerprints obtained for the aqueous and organic fractions were different and complementary (figure 2). These results were compared to the theoretical formic acid peptide fragments for Atxs A, B and C and sequence coverage was calculated to be 95.6%. However, it was not possible from these results to unambiguously identify the presence of all the individual Atxs. AtxB was confirmed by the presence of a unique peak at m/z 2851 and AtxC was confirmed by the presence of the distinctive peptide at m/z 1181. Unique peptides specific to AtxA, however overlapped with peptides from either AtxC (m/z 2853) or AtxB (m/z 1214) precluding unequivocal confirmation.

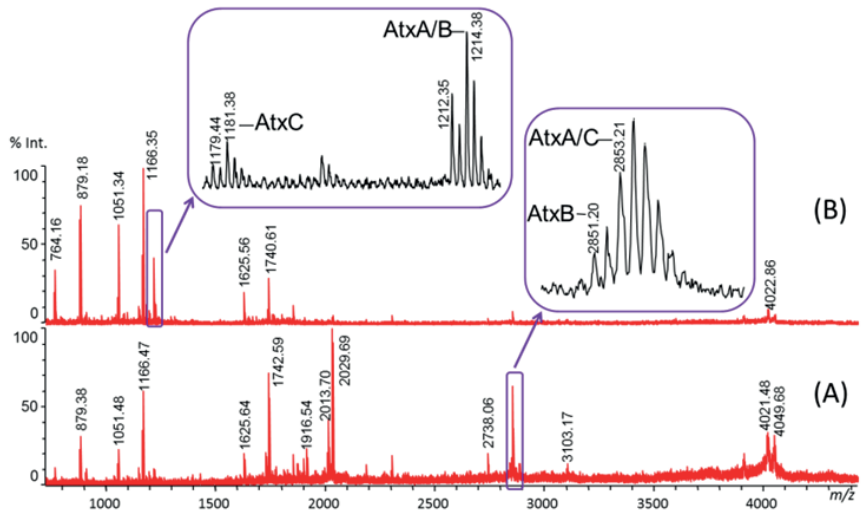

Figure 2: PMF of Atxs formic acid cleavage (A) aqueous ZipTip™ fraction, (B) organic ZipTip™ fraction

As anticipated, the most intense peaks observed in the PMF spectra corresponded to those resulting from cleavage at the carboxylic side of Asp residues. Also present in the spectra, but at a lower intensity, were peptides resulting from cleavage on the amino side of Asp, along with mixed cleavage (pre and post Asp) products, single formylation and water loss. Most interestingly, for peptides that contained an even number of cysteine residues, peaks 2 Da and 4 Da less that the peptide mass were observed (figure 3).

Further investigation of these particular peptides identified that these losses in fact correspond to disulfide bridge rearrangement of neighbouring cysteine residues. Although considered rare in proteins, this type of disulfide bridge is energetically possible and has been previously described in other toxins (1). Acidic conditions in the presence of DTT are usually effective in trapping the thiol –disulfide exchange, however it has been reported that in the case of rapid intra-molecular reactions, such as the vicinal cysteine rearrangement observed here, these conditions are inadequate (2). It is proposed in the Atxs case that although DTT was present in a sufficient concentration to effectively reduce all 7 disulfide bridges, oxidation may still occur during sample purification or drying as a result of highly reactive thiols or DTT degradation. The proposition of intra-molecular disulfide bridge formation was verified by investigation of the peptides formed through formic acid cleavage of reduced (DTT) and alkylated (iodoacetamide) Atxs. The peptide mass fingerprint obtained from this sample no longer exhibited the loss of 2 or 4 Da from peptides containing an even number of cysteine residues.

These results confirm the possibility of intra-molecular oxidation (disulfide bridge rearrangement) during formic acid cleavage under reducing conditions, a crucial experimental artefact that is rarely considered in the majority of studies.

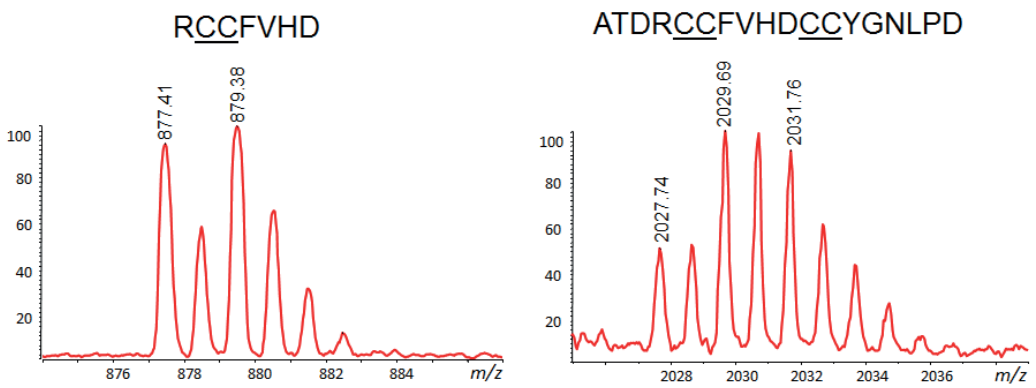

Figure 3: Example of Atxs peptides exhibiting loss of 2 or 4Da indicating one or two disufide bridges formed through oxidation.

Comparison of high and lower energy fragmentation of peptides containing intra-molecular disulfide bridges.

MS/MS investigation into the intra-molecular oxidised and reduced form of the Atxs peptide RCCFVHD was carried out by MALDI TOF-TOF in both PSD (low-energy) and high-energy CID modes to obtain structural confirmation and investigate differences observed between the two fragmentation modes.

PSD analysis of both the oxidised and reduced form of RCCFVHD exhibited the expected a-,b-, and y- type ions characteristic of lower energy fragmentation (figure 4). Abundant losses of NH_3 (17Da) from both a- and b- ion series were also observed and attributed to the presence of an arginine residue at the N-terminus of the peptide which directs fragmentation towards N-terminal ions. Furthermore, the a- and b-ion series for the oxidised form appear 2 Da lower in mass than the reduced form confirming the loss of two protons through intra-molecular reaction. The presence of an internal disulfide bridge was further corroborated by the absence of the a_2 ion, characteristic of amide bond cleavage between neighbouring cysteine residues. Furthermore based on the fragmentation pathway considered here (3) the ion observed at 651 m/z is most likely a result of an additional neutral loss (17Da) from the fragment ion a_6-NH_3. This proposition is further substantiated by the presence of a 653 m/z ion in the reduced peptide fragment spectrum (+2Da from two hydrogens). The ion formation timescale required for this process is coherent with the relatively lengthy time frame of PSD fragmentation.

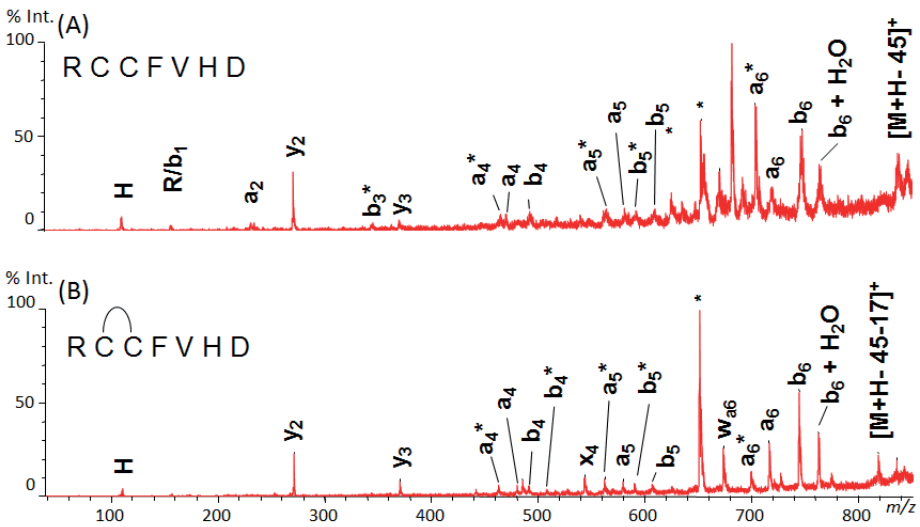

Figure 4: PSD analysis of the reduced (A) and oxidised (B) form of Atxs peptide: RCCFVHD

The oxidized and reduced forms of RCCFVHD were also investigated using true high-energy CID (20keV collision energy). Overall the fragment spectra exhibited more intense signals across the whole mass range (Figure 5) along with additional side-chain specific fragmentation pathways (d-type ions) particular to high-energy conditions. In addition to the a, b and y-ion series previously detected, the da_2 ion was observed a result of charge-remote intra-molecular hydrogen abstraction from the α-position of Cys (4). The reduced peptide also exhibited the da_3 ion as well as a_2 and a_3. In the oxidised species the formation of the a_2 is prevented by the rigid structure of the disulfide bridge. Finally as predicted the 651 and 653 m/z ions are absent from the high-energy CID fragmentation spectra due to the short timeframe of this process.

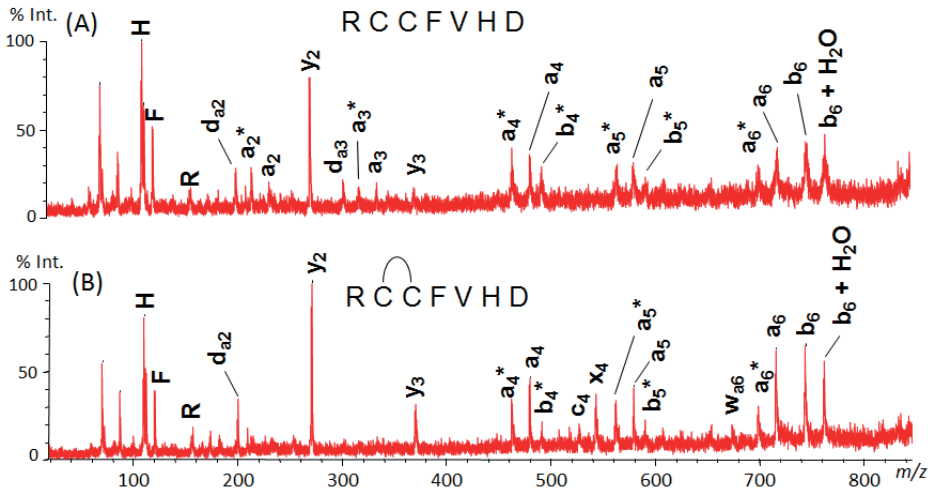

Figure 5: high-energy CID analysis of the reduced (A) and oxidised (B) form of Atxs peptide: RCCFVHD

Conclusions

- Formic acid cleavage of Atxs proteins treated with DTT results in good sequence coverage.
- For all investigated peptides, high-energy CID results in very good sequence characterization confirming Atxs amino acid sequence.
- Lower-energy PSD fragmentation are dominated by the typical a- b- and y-ions
- High-energy CID exhibits increased overall intensity of fragment ions across the mass range in addition of characteristic side chain fragmentation typified by the presence of d- and w-type ions.

Acknowledgements

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References

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