Towards a Complete Non-Linear Peptide MS/MS Characterization

Eva Duchoslav; Xu Guo; Tanmaykumar Desai
SCIEX, 71 Four Valley Drive, Concord, ON, L4K 4V8 Canada

ABSTRACT

This presentation highlights a generic workflow for characterization of non-linear peptides using high resolution MS/MS data. Non-linear peptides are represented as strings of amino acid residues with links outlining the attachment chemistry; this allows for the prediction of both single-chain and linked fragments. Comprehensive MS/MS annotation is possible by accommodating for side-chain losses as well as multiply-charged fragments and their isotope patterns in MS/MS. With this enhanced approach peptide sequence coverage and percent annotated MS/MS TIC were similar for data from IDA and DIA methods. The annotation coverage increased with the best outcome for purely cyclic peptides, where the improvements of up to 100% from the previous approaches[1,2] were noted.

INTRODUCTION

Biotherapeutic drugs are typically non-linear, cyclic peptides. These peptides and their catabolites need to be characterized within the drug development process.

While identification and annotation of linear peptides from MS/MS spectra has been well understood, the analysis of large bio-therapeutic peptides, such as cyclic ones, has presented challenges since their fragmentation does not follow conventional fragmentation pathways[3,4,5]. In addition, large bio-therapeutic peptides tend to generate larger, often multiply charged fragments and confident detection of the mono-isotopic ion can be difficult due to its potential absence or poor signal to noise. Meanwhile, the traditional naming logic for peptide fragments needs to be adjusted to fit non-linear sequences. This presentation will demonstrate the improvements of peptide annotation strategy addressing the above issues.

MATERIALS AND METHODS

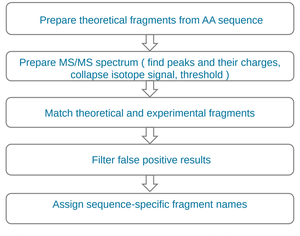
Sample Preparation:
Several non-linear peptide standards, oxytocin, cyclo(-GRGDSP), cyclosporine A and insulin (AnaSpec, 34801 Campus Drive, Fremont, CA 94555, USA) were prepared in 0.1% formic acid buffer and analyzed by LC/MS.

HPLC Conditions:
A Shimadzu Prominence LC system with an Agilent ZORBAX Eclipse Plus C18, 50x2.1mm, 1.8µm column at 40° C with a gradient of eluent A water + 0.1% Formic acid and eluent B acetonitrile+ 0.1% Formic acid was used at a flow rate of 200µL/min. The injection volume was set to 5µL.

MS Conditions:
Data was collected on a SCIEX X500B QTOF System with SCIEX OS 1.2. TOF MS acquisition covered m/z 100 to 1500 with 250ms accumulation time. IDA data were acquired using the peptide workflow using the charge state filter with dynamic background subtraction, intensity threshold 100 cps and maximum candidate ion 5. Data independent acquisition was performed using SWATH® Acquisition with 22 fixed fifty Da wide SWATH Acquisition windows. The MS/MS scan from each SWATH Acquisition covered m/z 100 to 1500 with a 100ms accumulation time. Total scan time for the SWATH Acquisition method was 2.5sec. Calibration delivery system (CDS) was used to calibrate the system between injections.

Data processing:
LC/MS data were processed with a research version of MetabolitePilot™ software. The sequences of non-linear peptides were provided in a string representation with the side–chain attachment descriptors. The non-natural amino acids were described in terms of their substructure building blocks, that were handled at the fragment level as variable modifications. In addition, characteristic fragments and neutral losses for amino acids from a peptide dictionary were utilized.

The processing workflow is outlined in Figure1.



Requested types of theoretical fragments are generated from a given sequence.

Experimentally observed neutral losses are validated against peptide structure and respective amino acids.

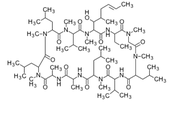
In case of isomeric fragments, the “winner” fragment requires the least bonds to break as well as evidence of a predecessor.

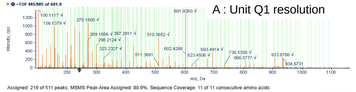
Figure 1: Workflow for the peptide MS/MS fragment annotation

RESULTS

Peptide: Cyclosporine A,
A[*1] L[1Me] L[1Me] V[1Me] T[_BMe] Abu G[1Me] L[1Me] V L[1Me] A[*1,(OH)-1]

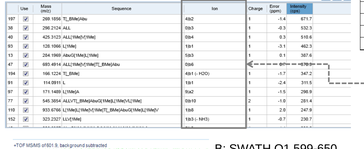
Abu: 2-Aminobutyric acid
[_BMe]: butenyl-methyl-
[1Me]: methyl
[*x, (OH)-1]: attachment of index x, loss of OH group from C-terminal



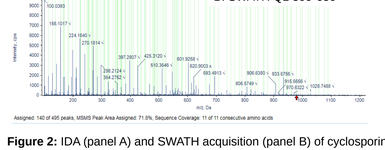


Comprehensive fragment annotation considers any combination of losses of peptide side chains. As such, the AL[1Me]L[1Me]V[1Me] fragment was found in 4 forms.

m/z	Fragment sequence	type	charge	error (ppm)	intensity
397.2807	AL1.V	0|b4	1	-0.6	3494.6
411.2962	AL|1Me|L.V	0|b4	1	-0.9	733.4
425.312	AL|1Me|L|1Me|V	0|b4	1	-0.6	2826.9
439.3275	AL|1Me|L|1Me|V|1Me|	0|b4	1	-0.9	258.8



Cyclic peptide fragment types indicate the AA index of the ring opening followed by a or b fragment of the resulting linear sequence.



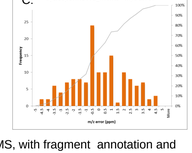
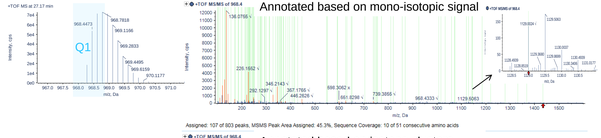


Figure 2: IDA (panel A) and SWATH acquisition (panel B) of cyclosporine MS/MS, with fragment annotation and distribution of m/z error, processed with a research MetabolitePilot™ software .

Peptide: Insulin, M=5729.6008

GIVEQC[*1]C[*2]ASV C[*1]SLYQLENYC[*3] N

FVNQHLC[*2]GSH LVEALYLVC[*3]G ERGFFYTPKA



Since series of isotope peaks are selected in Q1 and fragmented in parallel, peptide fragment isotopes in MS/MS spectra can be used in confident annotation.

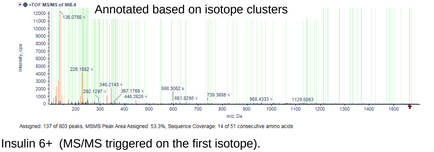


Figure 3: MS/MS of peptide Insulin 6+ (MS/MS triggered on the first isotope).

As biotherapeutic peptides tend to generate the large fragments, the monoisotopic ions are usually weak or, sometimes, absent. We investigated a number of approaches to handle this challenge by using the information from fragment isotope peaks; by collapsing fragment isotopic signal to signal of fragment mono-isotopic peak, we improve S/N of minor large fragments and enable their contribution to sequence confirmation. For example, in case of Insulin, the contribution of doubly charged fragments to the overall assignment rose from 3.6% to 6.9% .

Immonium ions (primary and secondary) are provided for the annotation from the amino acid dictionary. Cascading losses of water and ammonia are validated against the fragment type and structures of involved amino acid residues.

Peptide: Oxytocin, $C_{43}H_{66}N_{12}O_{12}S_2$, M = 1006.4365
C[*1]YIQNC[*1]PLG-[NH2]

m/z	Fragment	type	z	Error (mDa)	intensity
243.1098	QN	y6	b5	1	1	132.6
226.0829	QN	y6	b5 (-NH3 * 1)	1	0.6	217.3
209.0567	QN	y6	b5 (-NH3 * 2)	1	1	55.8
415.1753	QNCP	y6	a7	1	-0.5	23.6
398.1502	QNCP	y6	a7 (-NH3 * 1)	1	0.9	79.6
381.1235	QNCP	y6	a7 (-NH3 * 2)	1	0.7	104.6
443.1719	QNCP	y6	b7	1	1.2	30.2
426.1454	QNCP	y6	b7 (-NH3 * 1)	1	1.2	119.6
409.1183	QNCP	y6	b7 (-NH3 * 2)	1	0.7	38.9
556.2562	QNCPL	y6	b8	1	1.4	38.6
539.2294	QNCPL	y6	b8 (-NH3 * 1)	1	1.2	104.5
522.203	QNCPL	y6	b8 (-NH3 * 2)	1	1.4	32.1
630.3056	QNCPL-[NH2]	y6		1	2.8	23.9
613.2778	QNCPL-[NH2]	y6	(-NH3 * 1)	1	1.5	22.9
596.2512	QNCPL-[NH2]	y6	(-NH3 * 2)	1	1.5	31.8

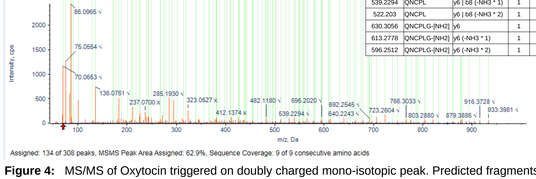


Figure 4: MS/MS of Oxytocin triggered on doubly charged mono-isotopic peak. Predicted fragments include breaking of the disulphide bond and cascading losses of ammonia from both side chains and C-terminus.

Peptide	Category	Sequence	Peaks (A)	Peaks (B)	Peaks (C)	TIC assigned (C)	Settings
Exenatide	Linear	HGEGTFTSDL SKQMEEEAVR LFIEWLKNGG PSSGAPPPS-[Ami]	25	57	174	43.6%	S/N >10 error< 5ppm
Oxytocin	Lasso	C[*1]YIQNC[*1]PLG-[NH2]	52	81	81	61%	S/N >10 error< 5ppm
Cyclo (-GRGDSP)	Cyclic	G[*1]RGDSP[*1,(OH)-1]	31	88	91	51.7%	S/N >10 error< 10ppm
Cyclosporin A	Cyclic with side chains	A[*1]L[1Me]L[1Me]V[1Me]T[_BMe]AbuG[1Me]L[1Me]VL[1Me]A[*1,(OH)-1]	160*	225	251	65.7%	S/N >10 Error< 5ppm
Insulin	Multi-chain	GIVEQC[*1]C[*2]ASV C[*1]SLYQLENYC[*3] N/ FVNQHLC[*2]GSH LVEALYLVC[*3]G ERGFFYTPKA	157	162	326	26.5%**	S/N >1000 error< 5ppm

Table 1. Summary of peptide interpretation results with different in-silico annotation strategies
Peaks (A) column indicates number of peptide MS/MS fragments having unique m/z, found considering a, b, y, y|a and y|b fragments; *Peaks (B) column* indicates number of peptide MS/MS fragments considering Peaks A and up to 4 side-chain losses; *Peaks (C) column* indicates number of peptide MS/MS fragments considering Peaks B and collapsing fragment isotope peaks to one peak.
*Variable side-chain methyl losses were considered.
**Fragments with up to 3 broken bonds were proposed in-silico.

Using the expanded algorithms, the number of annotated peptide fragments increased. The percentage of peak area assignment rose by a factor of 2.1 for peptide Oxytocin, and a factor of 2.0 for peptide Cyclo(-GRGDSP) compared to the previous annotation strategy.

CONCLUSIONS

- The workflow involving the SCIEX X500B QTOF System with a research version of MetabolitePilot™ software effectively utilizes complex peptide fragments leading to the characterization of non-linear peptides.
- The enhanced non-linear peptide representation can effectively describe various types of non-linear peptide sequences and their parts.
- With the implementation of non-natural amino acids and custom side-chain modifications into the amino-acid dictionary, the software exploits core of the cyclic structure as well as any side chain losses, thus improving fragment identification and sequence coverage.
- The enhanced algorithm recognizes fragment isotopic peaks and is well suited for leveraging information from larger multiply-charged fragments.
- The proposed workflow can be used not only for peptide sequence validation, but also for the detection, localization and characterization of modifications.

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