Evaluation of an automated, acidic pH protein digestion for reduced levels of artificial deamidation in biotherapeutic peptide mapping studies

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ABSTRACT

Assessment of the level of deamidation is essential for biotherapeutic characterization and development. pH conditions should be optimized during peptide mapping sample preparation to minimize artificially induced deamidation. The Thermo Scientific™ SMART Digest™ Kits (low pH, Magnetic Bulk Resin option) coupled with the Thermo Scientific™ KingFisher™ Duo Prime Purification System provide an optimized, automatable protein digestion approach that suppresses artificial deamidation events in biotherapeutic proteins during LC-MS peptide mapping studies.

INTRODUCTION

Deamidation is a chemical reaction that mainly occurs at asparagine (Asn/N) residues in proteins, but can also occur on Glutamine (Gln/Q)[1]. Typically, asparagine is converted to aspartic acid or isoaspartic acid via a succinimide intermediate. Assessment of the level of deamidation is essential for biotherapeutic characterization and development. A considerable body of literature suggests that alkaline pH conditions during protein digestion for peptide mapping analysis can induce deamidation[2-3].

Here, we investigate the potential of minimizing the level of deamidation induced during protein digestion using an automated magnetic bead-based sample preparation approach coupled with a novel optimized low pH digestion (Low pH Thermo Scientific™ SMART Digest™ kit) and subsequent analysis of native and modified peptides by Thermo Scientific Orbitrap™ based High-Resolution, Accurate-Mass (HRAM) Mass Spectrometry (MS).

MATERIALS AND METHODS

Protein Digestion
NIST Monoclonal Antibody Reference Material 8671 (10 mg/mL) was digested at a concentration of 0.5 mg/mL using the Thermo Scientific™ SMART Digest™ Trypsin Kit, Magnetic Bulk Resin option (P/N: 60109-101-MB-LPH) with the addition of 5 mM Thermo Scientific™ Bond-Breaker™ TCEP Solution (P/N: 77720) on the KingFisher Duo Prime Purification System (Catalog #: 5400100) for 30 minutes at 70 °C.

Table 1. The KingFisher DW 96 Plate (Catalog #: 95040450) set up

Lane	Content	Volume per well (µL)
A (Samples)	NISTmAb (2 mg/mL)	50
	Low pH SMART Digest™ Buffer	150
B (Tips)	12-tip comb for Microtiter 96 Deepwell plate (Catalog #: 97003500)	N/A
C	Blank	N/A
D (Resin)	SMART Digest™ Bulk Trypsin Magnetic Resin	15
	Low pH SMART Digest™ Buffer	100
E (Bead Wash)	Bead Wash Buffer (Low pH SMART Digest™ Buffer diluted 1:4 (v/v) in water, Optima™ LC/MS grade)	200
F (Waste)	Water, Optima™ LC/MS grade	200

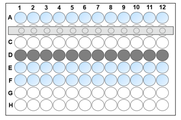


Figure 1 – KingFisher DW 96 Plate Layout

10-15 mins prep

KingFisher Duo Prime/Bindit Software method Parameters
The KingFisher Duo Prime Purification System was used to automate digestion, at 70 °C for 30 minutes. The parameters in Table 2 and Figure 2 were programed into Thermo Scientific™ BindIt™ Software (version 4.0).

Table 2. Protocol for automated peptide mapping using a KingFisher Duo Prime

Step	Release Beads	Mixing	End of Step	Temperature	Lane
Collect Beads	-	10 s (Bottom mix)	3 count, 1 s	-	D
Bead Wash	Yes	1 min (medium mix)	3 count, 1 s	-	E
Digest and Cool	Yes	30 min (medium mix)	3 count, 5 s	70 ° C Heating while Mixing 10 ° C Post Temperature	A
Release Beads	Yes, Fast	-	-	-	F
Leave (Tip comb)	-	-	-	-	B

Figure 2 –Bindit Software automated digest protocol

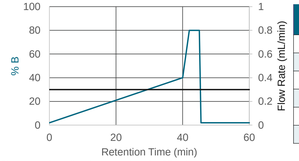
Figure 3 – KingFisher Duo Prime Purification System

30 mins digest

Liquid Chromatography Conditions
All digested samples were separated on a linear gradient, 2-40% acetonitrile with 0.1% formic acid (see Figure 4) using a Thermo Scientific™ Acclaim™ VANQUISH™ C18 column, 2.2 µm, 2.1 x 250 mm (P/N: 074812-V). For additional chromatographic conditions refer to Table 3.

Table 3. Chromatographic conditions for peptide mapping assessment

Column	Acclaim VANQUISH C18 column, 2.2 µm, 2.1 x 250 mm
Mobile Phase A	Water with 0.1% formic acid (v/v), Optima™ LC/MS grade (P/N: 10188164)
Mobile Phase B	Acetonitrile with 0.1% formic acid (v/v), Optima™ LC/MS grade (P/N: 10118464)
Flow Rate	0.3 mL/min
Column Temperature Settings	Column compartment: 60 °C Active pre-heater: 60 °C Post Column Cooler: 40 °C
Injection Volume	5 µL



Time (min)	%B
0.0	2.0
40.0	40.0
42.0	80.0
45.0	80.0
45.5	2.0
60.0	2.0

Figure 4 – LC gradient for separation of NISTmAb peptides

Mass Spectrometer Conditions:
Samples were analysed on a Thermo Scientific Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer. Conditions can be found in Tables 4 and 5.

Table 4. MS method settings or peptide mapping assessment

General	
Runtime	0 to 60 min
Polarity	Positive
Default Charge State	2
Full MS	
Resolution	140,000
AGC Target	3e6
Maximum IT	100 ms
Scan Range	200 – 1800 m/z
dd-MS2 / dd-SIM	
Resolution	17,000
AGC Target	1e5
Maximum IT	250 ms
Loop Count	5
TopN	5
Isolation Window	1.2 m/z
(N)CE/Stepped (N)CE	nce: 27
dd Settings	
Minimum AGC Target	2.00e3
Intensity Threshold	1.0e4
Charge Exclusion	unassigned
Peptide Match	preferred
Exclude Isotopes	on
Dynamic Exclusion	8.0 s

Table 5. MS tune settings for peptide mapping assessment

MS Source Settings	Value
Source	Ion Max source with HESI II probe
Sheath Gas	35 arb
Aux Gas	10 arb
Sweep Gas	0 arb
Spray Voltage	3.5 kV
S-lens RF Level	50
Aux Gas Temp	250 °C
Capillary Temp	250 °C

60 mins run time

MS Data Processing:
Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) Software version 7.2.9 (Cat. No. CHROMELEON7) was used for data acquisition and analysis. For data processing, Thermo Scientific™ Biopharma Finder™ software version 3.1 was used.

RESULTS

The results show a significant reduction in the level of relative deamidation in low pH SMART Digest prepared samples when compared to conventional SMART Digest buffer control digests. When using the modification summary in BioPharma Finder for comparison, reductions in deamidation were observed at residues ~N206, N289, N318, N328, N364 and ~N437 in the heavy chain of NISTmAb (Figure 5). There is an average 0.55% reduction in relative % deamidation for Asn residues when using the low pH SMART Digest Kits (Table 6).

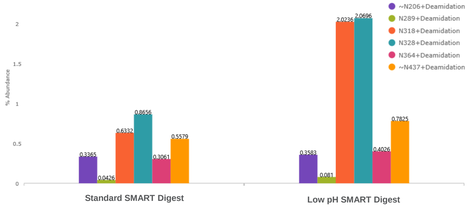


Figure 5– NISTmAb deamidation modification plot in BioPharma Finder

Table 6. % Relative Asn deamidation comparison

Asparagine (N) Site	Standard SMART Digest	Low pH SMART Digest
N206	0.36	0.34
N289	0.08	0.04
N318	2.02	0.63
N328	2.07	0.87
N364	0.40	0.31
Total (%):	**4.93**	**2.19**
Average (%):	**0.99**	**0.44**

Upon further investigation, it was noted that the site which showed the biggest reduction in relative deamidation (1.39 %) was N318 in the NISTmAb Heavy Chain. This is of particular interest as within the peptide sequence (V305-K320) the adjacent residue to N318 is a Glycine (G) VVSVLTVLHQDWL**NGK**. The N+1 residue is particularly important, because its size and charge influences the local flexibility of the peptide backbone; a G residue at N+1 has the greatest effect on the rate of deamidation, followed by histidine (H) and Serine (S)[4]. MS/MS detection was used as further confirmation of the deamidation sites. Deamidation results in a 0.984 Da increase in mass when comparing to the unmodified precursor ion (see Figure 6).

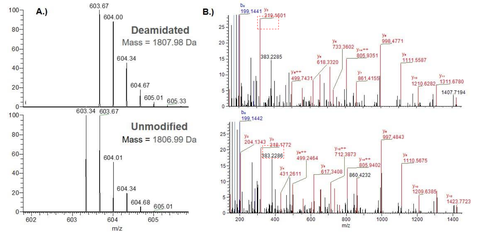


Figure 6 – (A) MS and (B) MS/MS Spectra of deamidated and unmodified peptides (VVSVLTVLHQDWLNGK)

A comparison of the standard SMART Digest vs the low pH SMART Digest Base Peak (BP) chromatograms was made to show the profiles were comparable (Figure 7). The reduction in overall BP intensity observed is likely to be attributed to the lower pH reducing the activity of the Trypsin protease[5].

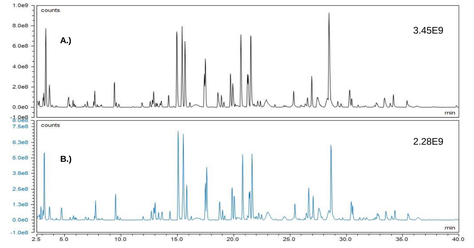


Figure 6 – BP Chromatogram (A) Standard SMART Digest, (B) Low pH SMART Digest from Chromeleon CDS 7.2.10

To ensure that there were no detrimental effects to quality of the data at the lower pH, an assessment of the % sequence coverage in BioPharma Finder was made. Comparable levels of sequence coverage was observed for both the heavy and light chains of NISTmAb using the low pH SMART Digest Kits (Table 7).

Table 7. % BPF 3.1 Analysis of NISTmAb Sequence Coverage

Low pH SMART Digest Kit			
	Number of MS Peaks	Sequence Coverage	Abundance (mol)
NISTmAb LC	381	100%	33.68%
NISTmAb HC	1034	100%	66.32%

Standard SMART Digest Kit			
	Number of MS Peaks	Sequence Coverage	Abundance (mol)
NISTmAb LC	371	100%	28.97%
NISTmAb HC	1009	99.6%	71.03%

CONCLUSIONS

- When using the new low pH SMART Digest Kit a reduced amount of relative % deamidation of Asparagine residues is observed compared to the standard Smart Digest Kits.
- A significant reduction in the relative % deamidation was observed even for 'NG' motif peptides which are more susceptible to neutrophilic attack.
- As expected, the overall peptide intensity is marginally reduced when using the low pH kit compared to the standard SMART digest kit, however the impact to sequence coverage and data quality is minimal.
- Magnetic SMART Digest Kits, coupled with the KingFisher Duo Prime purification system, provide a reproducible automated approach to protein digestion.

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TRADEMARKS/LICENSING

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