Poster Note 21211

Generation of a Novel Digestion Protocol for Enhanced Proteome Coverage

Valeria Barattini, Phillip Humphryes, Jon Bardsley, Joanne Jones, and Tim Liddicoat
Thermo Fisher Scientific, Runcorn, UK

Overview

Here we present a novel approach for tryptic digestion using the Thermo Scientific™ SMART Digest™ kit , an immobilized, heat-stable trypsin with an optimized buffer system. This results in rapid and precise digestions, removing uncertainty associated with conventional solution-based protocols.

The SMART Digest kit was used in the analysis of thyroglobulin biomarkers in murine plasma, where complete digestion was achieved in 3 hours. Three unique peptides were selected for comparison to conventional solution-based protocols previously published[1]. The signal response was increased 2-fold with our immobilised trypsin digestion protocol. A calibration curve for one of the selected peptides was then prepared to quantify thyroglobulin in plasma.

Introduction

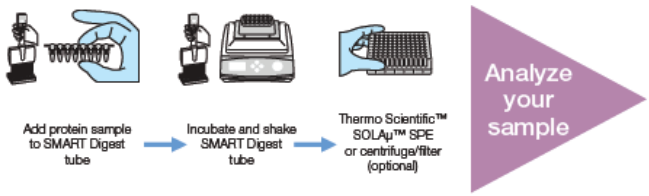
Protein digestion is a fundamental technique employed in biopharmaceutical and proteomic applications. Despite its widespread use, protein digestion still provides many analytical challenges. The optimum digestion requires conditions that:

- Cleave the proteins after every lysine and arginine
- Unfold the protein/proteins of interest
- Reduces autolysis effects

This process can be lengthy and complex and often leads to poor reproducibility.

The SMART Digest kit removes uncertainty associated with conventional solution-based tryptic digestion protocols. The immobilized trypsin technology, combined with the optimized buffer result in higher reproducibility and superior sample characterization (Figure 1).

FIGURE 1. Simplified digestion protocol using the SMART Digest kit.



In this study we present the SMART Digest kit as a tool for the digestion of thyroglobulin in murine plasma. The first set of experiments defined the digestion time, then a calibration curve was prepared using unique peptides identified through mapping studies. In the second set of experiments, a previously published solution-based assay protocol was reproduced and the results compared.

Methods

Experiment 1:

Digestion time optimization

20 µg of thyroglobulin were added to each tube containing 150 µL SMART Digest buffer and 50 µL murine plasma. These were incubated at 70 °C with 1400 rpm shaking. Wells were sampled every 30 minutes and diluted 10-fold in Tris buffer saline (TBS) before analysis.

Calibration curve preparation

Different concentrations of thyroglobulin were prepared in SMART Digest tubes containing 150 µL SMART Digest Buffer and 50 µL of murine plasma. A concentration range of 4–4000 µg/mL was covered. These were incubated at 70 °C and 1400 rpm. Samples were collected at 210 minutes and diluted 10-fold in TBS before analysis.

Thermo SCIENTIFIC

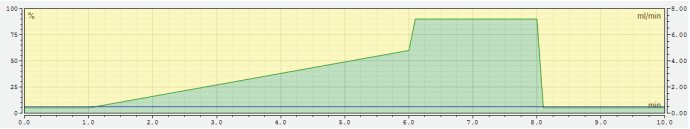
Experiment 2:
Comparison to Standard Method[1]
Samples of 50 µL murine plasma were spiked with 4 µg/mL of thyroglobulin, diluted with 0.2 mL of reduction buffer and reduced for one hour at 37 °C. Samples were then alkylated before incubation at room temperature for one hour. 3 µg of sequencing grade trypsin were added and the samples incubated for four hours at 37 °C with agitation. A further 10 µg of sequencing grade trypsin were added and the sample incubated for 16 hours at 37 °C with agitation. Samples were diluted with water 10-fold prior to analysis.

Sample Preparation

SMART Digest Kit

Liquid Chromatography
Mobile phases:
A: 2% Acetonitrile, 97.9% water, 0.1% Formic Acid
B: 90% Acetonitrile, 9.9% water, 0.1% Formic Acid

Gradient:



Column: Thermo Scientific™ Accucore™ C18 (50 x 2.1 mm)
Column temperature: 40 °C
Flow rate: 0.5 mL/min

Instrumentation
Samples were analyzed on a Thermo Scientific™ Dionex ™ UltiMate™ 3000 RSLC, coupled with a Thermo Scientific™ Velos Pro™ ion trap mass spectrometer.

Data Analysis
Thermo Scientific™ Xcalibur™ 2.0, Qual browser version 2.0.3.2.

Results

Experiment 1: Digestion time optimization

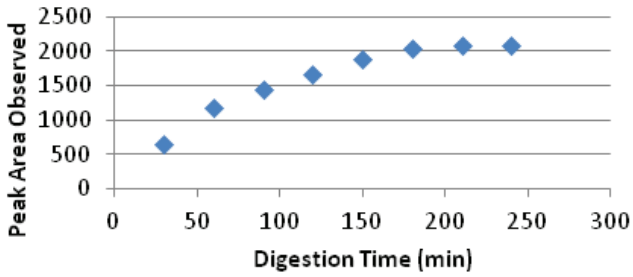
Three well characterized peptides were chosen for our initial screening studies. The SRM transitions for each selected peptide are listed in Table 1.

TABLE 1. SRM transitions for monitored peptide sequences of thyroglobulin.

Peptide Sequence	Precursor Ion (*m/z*)	Fragment Ion (*m/z*)
VIFDANAPVAVR	636.5	541.3
LGDQEFIK	475.4	836.4
FPLGESFLVAK	604.5	850.4

Complete digestion was achieved within 210 min using the SMART Digest kit. Figure 2 shows the trend observed for the peptide LGDQRFIK with an m/z of 475.4. This trend was confirmed for the other selected peptides (data not shown).

FIGURE 2. Digestion time course of thyroglobulin monitoring the generation of peptide LGDQEFIK.

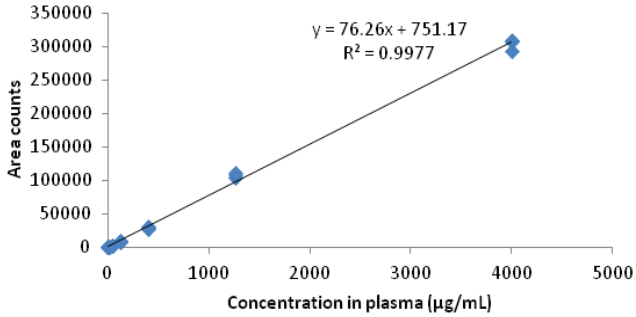


A calibration curve (Figure 3, Table 2) was produced to determine the LLOQ of thyroglobulin in a plasma matrix (n=3); additional peptides were also assessed which showed similar results (not shown). Whilst conventional methods would involve an immune-affinity purification to increase sensitivity this was not required using the SMART Digest kit.

Table 2. Calibration data for the LGDQEFIK peptide in murine plasma (n=3).

Concentration (µg/ml)	CV
0.00	0
4.00	17.4
12.64	22.1
40.00	4.8
126.40	9.0
400.00	3.6
1264.00	3.1
4000.00	3.0

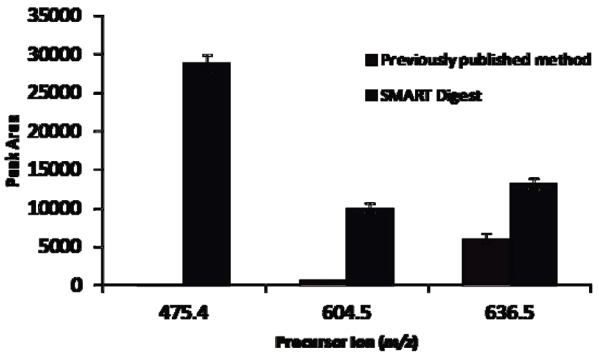
FIGURE 3. Graphical representation of the calibration curve for thyroglobulin in murine plasma using the LGDQEFIK tryptic peptide.



Experiment 2: Comparison to standard method[1]

The peptides listed in Table 1 were monitored in samples generated from in-solution based digestion. For all three peptides studied, the SMART Digest kit displayed double the signal response than the standard in solution digestion method assessed.

FIGURE 4. Comparison of peak area intensities (at 500 µg/mL) for three biomarker peptides from digested thyroglobulin using SMART Digest kit protocol and a previously published in-solution based digestion protocol[1].



The peptide LGDQEFIK (m/z 475.4) was undetectable at a concentration of 500 µg/mL in plasma (Figure 4), using the in solution method.

Conclusion

The SMART Digest kit allows increased biomarker identification by providing a fast, simple and reproducible tryptic digestion protocol:

- Complete digestion of thyroglobulin from whole murine plasma was achieved in 3.5 hours, a significant time saving compared to the 22 hours required for in-solution based protocols.
- A calibration line was prepared showing excellent linearity and reproducibility in a range of 4–4000 µg/mL.
- Comparison with previously published data showed at least double signal intensities for all three peptides selected.

References

1. Clarke, N J, et al, A novel mass spectrometry-based assay for the accurate measurement of thyroglobulin from patient samples containing antithyroglbulin antobodies, *Journal of Investigative Medicine*, **2012**, 60, 1157-1163.

For research use only

For more information, visit our website at **www.thermoscientific.com/chromatography**

USA and Canada +1 800 332 3331
Australia 1300 735 292 (free call domestic)
China 800 810 5118 (free call domestic)
400 650 5118
France +33 (0)1 60 92 48 34
Germany +49 (0) 2423 9431 20 or 21
India +91 22 6742 9494
+91 27 1766 2352
Japan 0120 753 670 (free call domestic)
0120 753 671 (fax)
Korea +82 2 3420 8600
United Kingdom +44 (0) 1928 534 110
New Zealand 0800 933 966 (free call domestic)
Singapore +65 6289 1190
All Other Enquiries +44 (0) 1928 534 050

Technical Support

For advice and support, please visit our website: www.thermoscientific.com/chromexpert

Thermo SCIENTIFIC
A Thermo Fisher Scientific Brand

PN21211-EN 0515S