Application Note 21180

Enhanced Digestion for Improved Biomarker Identification

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Key Words

Peptides, proteins, thyroglobulin, monoclonal antibody, mAb, biomarker, SMART Digest, protein digestions, proteomics, Accucore C18, biopharmaceutical, biomolecules, biotherapeutics

Goal

To demonstrate how the Thermo Scientific™ SMART Digest™ kit makes it possible to quickly digest even challenging biomarkers such as thyroglobulin, while significantly improving sensitivity over previously described methods.

Introduction

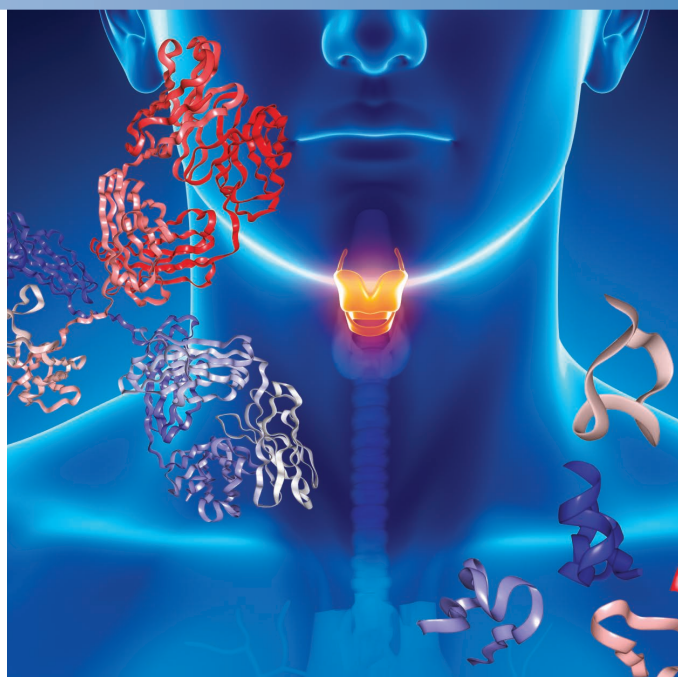
Biomarker identification is analytically challenging due to sample complexity and inherently low levels of many important biomarkers. Clean-up and enrichment strategies have resulted in significant gains in analytical sensitivity. However, complete digestion of the protein can also improve sensitivity and generate more peptides.

Biomarkers digest at different rates based on their properties; some are completely digested in a few hours while others are only partially digested after 48 hours.[1] Incomplete digestion leads to decreased sensitivity and sample to sample variability.

Protein digestion is a fundamental technique employed in biopharmaceutical and proteomic applications. It is used to analyze the sample and post translational modifications (PTMs). Despite its widespread use, protein digestion still provides many analytical challenges. The optimum trypsin digestion should provide conditions that accomplish the following:

- Cleave the proteins after every lysine and arginine
- Unfold the protein/proteins of interest
- Completely denature the protein to be digested yet not affect trypsin itself
- Use the minimum amount of trypsin to protein ratio to prevent partial digestion

Thyroglobulin is a large protein of significant interest. However, it is difficult to digest and the current methodology is unreliable. This protein provides an opportunity to establish the viability of new digestion techniques to improve speed and sensitivity.



Currently, the most reliable method of determining thyroglobulin levels is to perform a protein digestion of the plasma, followed by affinity purification using anti-peptide antibodies with subsequent analysis by liquid chromatography/mass spectrometry system. In this way, the sample complexity can be reduced without interference from native anti-thyroglobulin antibodies.

The inherent difficulty in this workflow is the limited ability to efficiently obtain reproducible and accurate results when digesting proteins from a complex matrix. Here we present a method for performing the digestion of thyroglobulin in an efficient and reproducible manner using an immobilized, thermally stable form of trypsin. The SMART Digest kit removes uncertainty associated with conventional solution-based tryptic digestion protocols, resulting in higher reproducibility and sample characterization.

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In the first set of experiments, peptide mapping of thyroglobulin was run to identify the biomarkers of interest. These were chosen based on previously published results.[2] After these peptides were identified, time-course studies were run to optimize the digestion time of thyroglobulin in murine plasma. Finally, a calibration curve was measured for digested samples with no further treatment.

In the second set of experiments a previously published in-solution digest protocol[2] was followed and the results compared with data from SMART-digested samples.

Experimental

Digestion

- SMART Digest Kit (P/N 60109-101)

Chemicals

- Fisher BioReagents™ tris buffered saline (TBS) (P/N 10648973)
- Fisher Scientific™ Optima™ LC-MS Water (P/N 10095164)
- Fisher Scientific Optima acetonitrile (ACN) (P/N 10001334)
- Fisher Scientific Optima isopranol (IPA) (P/N 10091304)
- Fisher Scientific dimethyl sulfoxide (DMSO) (P/N 10500151)
- Fisher Scientific Optima methanol (MeOH) (P/N 10031094)
- Fisher BioReagents Tween 20 (P/N 10113103)
- Thermo Scientific™ Pierce™ octyl-beta-glucoside (P/N 28310)
- Fisher Scientific Optima formic acid (P/N 10596814)
- Fisher BioReagents ammonium bicarbonate (P/N 10532775)
- Fisher BioReagents dithiothreitol (P/N 10386833)
- Acros Organics™ iodoacetamide (P/N 10346660)
- Pierce trypsin protease, MS grade (P/N 13454189)
- Pierce octyl-beta-glucoside (P/N 28310)
- Fisher BioReagents tris buffered saline (TBS), 10X solution, pH 7.4 for molecular biology (P/N 10153103)
- Human thyroglobulin and murine plasma were purchased from reputable sources.

Sample Handling Equipment

NSC Mass Spec Certified 2 mL clear vial with blue bonded PTFE silicone cap MSCERT4000-34W

Heater/shaker equipped with PCR block and heated lid

Separation

Thermo Scientific™ Accucore™ C18 column (50 × 2.1 mm, 2.6 µm particle) (P/N 17126-052130)

Experiment 1: Digestion time optimization and calibration curve measurement

Thyroglobulin peptide mapping

Samples were prepared by adding 20 µg of thyroglobulin to each well containing 150 µL SMART Digest buffer, 50 µL of water, and 0.1 wt% octyl-β-glucoside. These were incubated at 70 °C and 1400 rpm for 2 hours before analysis.

Digestion

Samples were prepared by adding 20 µg of thyroglobulin to each well containing 150 µL SMART Digest buffer and 50 µL murine plasma. These were incubated at 70 °C and 1400 rpm. Wells were sampled every 30 minutes and diluted 10-fold in tris buffer saline (TBS) before analysis.

Calibration curve sample preparation

Samples were prepared to cover a concentration range of 4–4000 µg/mL. The thyroglobulin was spiked in various weights into each well of three SMART Digest strips containing 150 µL SMART Digest buffer and 50 µL murine plasma. These were incubated at 70 °C and 1400 rpm. Samples were collected at 210 minutes and diluted 10-fold in TBS before analysis.

Experiment 2: In-solution digestion protocol

Comparison to existing methods[2]

Reduction buffer was made containing 125 mM NH_3HCO_3, 0.0086% Tween 20, and 12.5 mM dithiothreitol. Samples of 50 µL of murine plasma were spiked with 4 µg/mL of thyroglobulin to match the high end of the linearity tests performed with the SMART Digest kit and then diluted with 0.2 mL of reduction buffer before being reduced for 1 hour at 37 °C with agitation. Samples were then alkylated by the addition of 5 µL of 200 mM iodoacetamide before incubation in the dark at room temperature for 1 hour. At this point, 3 µg of sequencing grade trypsin was added and the samples were incubated for 4 hours at 37 °C with agitation. Subsequently, an additional 10 µg of sequencing grade trypsin was added and the sample was incubated for another 16 hours at 37 °C with agitation. Samples were diluted 10-fold prior to analysis.

Separation Conditions

Instrumentation	Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system interfaced with a Thermo Scientific™ Velos Pro™ ion trap mass spectrometer
Mobile phase A	2% acetonitrile, 98% water, 0.1% formic acid
Mobile phase B	90% acetonitrile, 10% water, 0.1% formic acid
Gradients	LC Gradient 1 (Table 1) was used in the peptide mapping experiments. All other analyses were run using Gradient 2 (Table 2).
Flow rate	0.5 mL/min
Column temp.	40 °C
Injection details	5 µL (for peptide mapping and calibration curve development) 2 µL (for digestion time course analysis)

Table 1. LC Gradient 1 conditions.

Time (min)	% A	% B
0	98	2
1	98	2
61	50	50
61.1	10	90
63	10	90
63.1	98	2
65	98	2

Table 2. LC Gradient 2 conditions.

Time (min)	% A	% B
0	95	5
1	95	5
6	40	60
6.1	10	90
8	10	90
8.01	95	5
10	95	5

Software

Thermo Scientific™ Xcalibur™ software 2.0, Qual browser version 2.0.3.2

Results and Discussion

Three peptides were selected for study. These were chosen on initial screening results as well as literature surveys. The SRM transitions for each selected peptide are listed in Table 3.

Table 3 Monitored peptide sequences for thyroglobulin.

Peptide Sequence	Precursor Ion *(m/z)*	Fragment Ion *(m/z)*
VIFDANAPVAVR	636.5	541.3
LGDQEFIK	475.4	836.37
FPLGESFLVAK	604.5	850.4

Running a digestion time course, a maximum sensitivity was obtained following 210 minutes of digestion using the temperature stable immobilized enzyme. Figure 1 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).

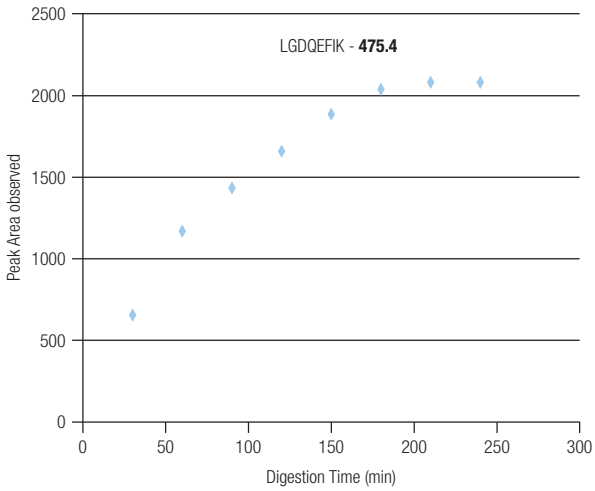

Figure 1. Digestion time course of thyroglobulin examining the generation of the LGDQEFIK peptide.

Following the time course analysis, a calibration curve was run to determine an approximate LLOQ of thyroglobulin in a plasma matrix. Triplicates were run for statistical accuracy. While conventional methods would involve an immune-affinity purification to increase sensitivity, no further sample preparation was applied after digestion.

The concentration range of thyroglobulin was from 4 µg/mL to 4 mg/mL in plasma. The peptide VIFDANAPVAVR is commonly used for thyroglobulin quantitation. Initial studies on this peptide showed it to have a good signal at the high end of the calibration curve; however, this was also accompanied by low S/N values. The peptide LGDQEFIK was chosen as an alternative biomarker. This peptide showed strong linearity through the entire calibration curve (Figure 2), with CVs <10% down to 40 µg/mL, below which instrumentation variance becomes a significant factor (Table 4).

Table 4. Calibration curve for the LGDQEFIK peptide in murine plasma.

LGDQEFIK - 475.4/836.37 Calibration Curve in Murine Plasma						
Concentration (µg/mL)	**Rep1**	**Rep2**	**Rep3**	**Average**	**StDev**	**CV**
0	0	0	0	0	0	0
4	330	247	347	308.0	53.5	17.4
12.64	951	771	608	776.7	171.6	22.1
40	2696	2876	2963	2845.0	136.2	**4.8**
126.4	9303	9476	8010	8929.7	801.1	**9.0**
400	29911	27844	29048	28934.3	1038.2	**3.6**
1264	103781	107545	110303	107209.7	3273.9	**3.1**
4000	292450	307302	308959	302903.7	9091.0	**3.0**

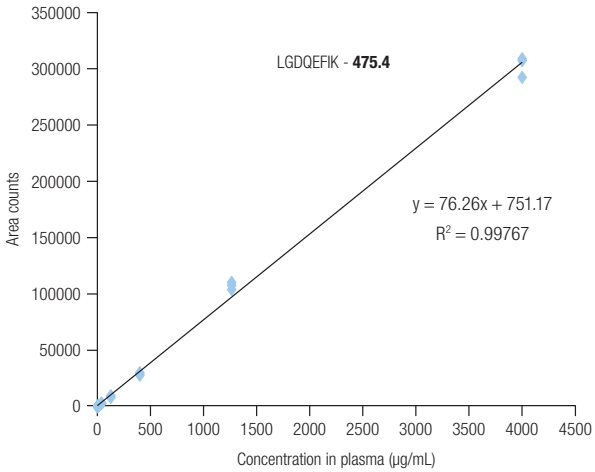

Figure 2. Calibration curve for thyroglobulin in murine plasma using the LGDQEFIK peptide.

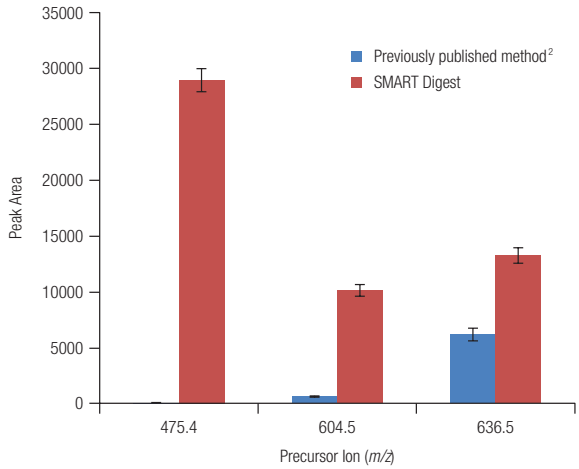

Figure 3. Comparison of the SMART Digest protocol with a previously published in-solution digestion protocol.[2]

Comparison to previously published methods[2] using in-solution digestion showed less than half the signal response for each of the three peptides studied, with LGDQEFIK being entirely undetectable at a concentration of 500 µg/mL in plasma (Figure 3).

The well-known limitations of conventional digestion protocols suggest that the lower signal achieved by in-solution digestion of thyroglobulin is largely due to incomplete lysis of the protein. This possibility is particularly troublesome in a quantitative assay. Even though peptide internal standards are able to offset sample to sample variation due to matrix effects, they are not able to account for the variance due to poor digestion efficiency. This will significantly influence the results if digestion is only partially completed.

Conclusion

The SMART Digest kit allows increased biomarker identification by providing a fast, simple, and clean tryptic digestion protocol. A comparison between the SMART Digest protocol and published solution-based digestion protocol was run for the quantitation of thyroglobulin from whole murine plasma. Complete digestion was achieved in 3.5 hours with a SMART Digest kit, compared to 22 hours required for the solution-based protocol. The signal response of key SRM-peptides was found to be doubled when using the SMART Digest kit. Excellent linearity was observed for the key SRM thyroglobulin peptides.

References

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2 Clarke, N.J.; Zhang, Y.; Reitz, R.E. A novel mass spectrometry-based assay for the accurate measurement of thyroglobulin from patient samples containing antithyroglobulin autoantibodies, *Journal of Investigative Medicine*, **2012**, 60, 1157-1163.

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