

Fast Digestion Method Optimization Using Innovative Trypsin Technology

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Key Words

SMART Digest kit, peptides, monoclonal antibody, protein, bottom-up proteomics, proteomics, trypsin digestion, buffer, mAb, biomolecules, biotherapeutics, biopharmaceutical, biocompatible UHPLC, MabPac RP, peptide mapping, in-solution digestion, ribonuclease A

Goal

To describe how to construct a simple time course experiment to identify the digestion endpoint for a given protein by monitoring degradation of the intact protein. The method is simple and uses LC-UV detection. Ribonuclease A, a small, heat stable protein, and mouse IgG, a large immunoglobulin, are used as test probes.

Introduction

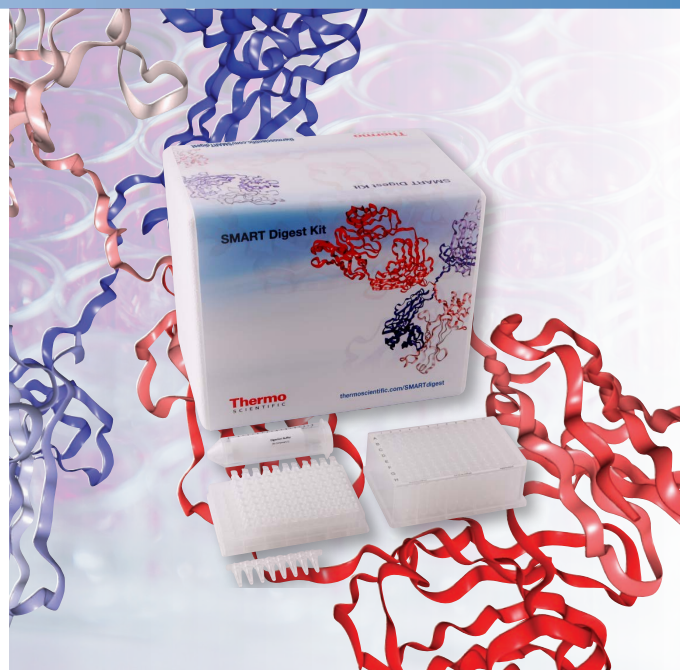
Standard in-solution protocols for tryptic digestion are lengthy, multifaceted, and prone to irreproducibility. Thermo Scientific™ SMART Digest™ Kits eliminate these issues by providing a protocol that is:

- Highly reproducible
- Quick and easy to use
- Detergent free
- Highly amenable to automation

When working with a new target protein, it is important to identify the optimum SMART digestion time to achieve complete digestion. A simple way to identify this is to monitor the disappearance of the intact protein from the sample by LC-UV. It is crucial that an appropriate stationary phase is selected.

The Thermo Scientific™ MAbPac™ RP column is a polymeric reversed-phase (RP) LC column designed for monoclonal antibody (mAb) characterization including separation of intact mAbs, mAb fragments, and large biomolecules. The unique column chemistry provides excellent performance under a broad range of pH, temperature, and mobile phase composition.

Here we show proof of principle experiments on how to identify the optimum SMART digestion time using ribonuclease A and mouse IgG as test probes. Ribonuclease A is a small, heat stable protein with a molecular weight of 13.7 kDa, and IgG is an antibody of



approximately 150 kDa molecular weight. While the two proteins differ largely in size, their digestion presents a number of challenges. Ribonuclease A is particularly resistant to tryptic digestion due to its heat stability and ability to quickly refold,¹ while IgG presents a considerable challenge due to its large size.

Digestion progress using the SMART Digest kit is monitored to completion by running a simple time-course experiment and monitoring the disappearance of the intact protein and the stability of the peptides produced. The method is simple and robust and applicable to any extracted target protein sample.

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Experimental

Digestion

- SMART Digest kit with 96-well filter plate (P/N 60109-102)

Chemicals

- Deionized water, 18.2 M Ω /cm resistivity
- Fisher Scientific™ Optima™ acetonitrile (ACN) (P/N 10001334)
- Fisher Scientific™ trifluoroacetic acid (TFA) (P/N 10294110)
- Mouse IgG Isotype Control (3 mg/mL solution) (P/N 10400C)

Ribonuclease A from bovine pancreas was purchased from a reputable source.

Sample Handling

- 2 mL Chromatography Certified 96-well square plates (P/N 60180-P202)
- Thermo Scientific™ WebSeal™ Silicone/PTFE mats (P/N 60180-M122)

Sample Handling Equipment

- 96-well positive pressure manifold (P/N 60103-357)

It is also recommended that a heater/shaker equipped with PCR block be used to perform the digestion.

Sample Pretreatment

Ribonuclease A

To each SMART Digest trypsin tube 75 μ L of SMART buffer were added. Ribonuclease A was subsequently added in 25 μ L aliquots (2 mg/mL, 50 μ g). To ensure a uniform starting point across all the tubes, the aliquots were added using an 8-channel pipette. Samples were digested using a heater shaker set at 70 °C and 1400 rpm shaking. Tubes were subsequently removed after 15, 30, 60, 90, 120, 150, 180, and 210 minutes. Each time point was prepared in triplicate.

Mouse IgG

To each SMART Digest trypsin tube 75 μ L of SMART buffer were added in 25 μ L aliquots (3 mg/mL, 75 μ g). To ensure a uniform starting point across all the tubes, the aliquots were added using an 8-channel pipette. Samples were digested using a heater shaker set at 70 °C and 1400 rpm shaking. Tubes were removed after 15, 30, 45, 60, 90, 120, 150, and 180 minutes. Each time point was prepared in triplicate.

For both sample sets, at the required time point digestion was stopped by simply forcing the sample through the filter plate using positive pressure to remove any immobilized trypsin beads. The eluent was analyzed using LC-UV.

Samples corresponding to t=0 minutes were prepared by diluting 25 μ L of ribonuclease A or IgG solutions with 75 μ L of SMART buffer. No mixing with trypsin beads was applied. The whole sample was then forced through the filter plate using positive pressure.

Separation Conditions

Instrumentation	Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system equipped with:
	SRD-3600 Solvent Racks with Degasser (P/N 5035.9230)
	DGP-3600RS Rapid Separation Pump (P/N 5040.0066)
	WPS-3000TRS Rapid Separation Thermostatted Autosampler (P/N 5841.0020)
	TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
	DAD-3000RS Rapid Separation Diode Array Detector (P/N 5082.0020)
Column	MabPac RP, 50 \times 2.1 mm (P/N 088648)
Mobile Phase A	Water + 0.1 % TFA
Mobile Phase B	Acetonitrile + 0.08% TFA
Gradient	See Table 1
Flow Rate	0.5 mL/min
Column Temperature	80 °C
Autosampler Settings:	The autosampler pick-up settings were optimized for this analysis. Settings described in Table 2 were applied. These settings may differ depending on the autosampler type and manufacturer.
Injection Wash Solvent	Water + 0.1% TFA
UV Detector	Wavelengths selected were 214 nm and 280 nm. Peak width was set to 0.1 min and recommended values were selected for the data collection settings.

Table 1. LC gradient conditions.

Time	%B	Curve
0	0	5
1	0	5
11	50	5
11.01	100	5
15	100	5
15.01	0	5
20	0	5

Table 2. Autosampler settings.

Parameter	Setting
Injection Volume	5 μ L
Draw Speed	0.05 μ L/s
Draw Delay	3.0 s
Dispense Speed	10.0 μ L/s
Dispense Delay	2.0 s
Dispense to Waste Speed	1.0 μ L/s
Sample Height	2.0 mm

Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ 7.2 SR2 Chromatography Data System was used for data acquisition and analysis.

Results and Discussion

The MAbPac RP column is packed with supermacroporous polymer resin. Its large pore size (~ 1,500 Å) is suitable for separation of large proteins. In addition, large biomolecule analysis by reversed-phase liquid chromatography requires high temperatures such as 70 to 80 °C. The polymeric nature of the MAbPac RP column provides both pH and temperature stability, making it an ideal column for protein analysis.

First, a separation method was developed for intact ribonuclease A and IgG. A single peak was identified for both analytes (Figure 1).

Digested and filtered ribonuclease A and IgG samples were subsequently analyzed with the same method. To monitor digestion progress, the chromatograms of each time point were overlaid (Figures 2 and 3).

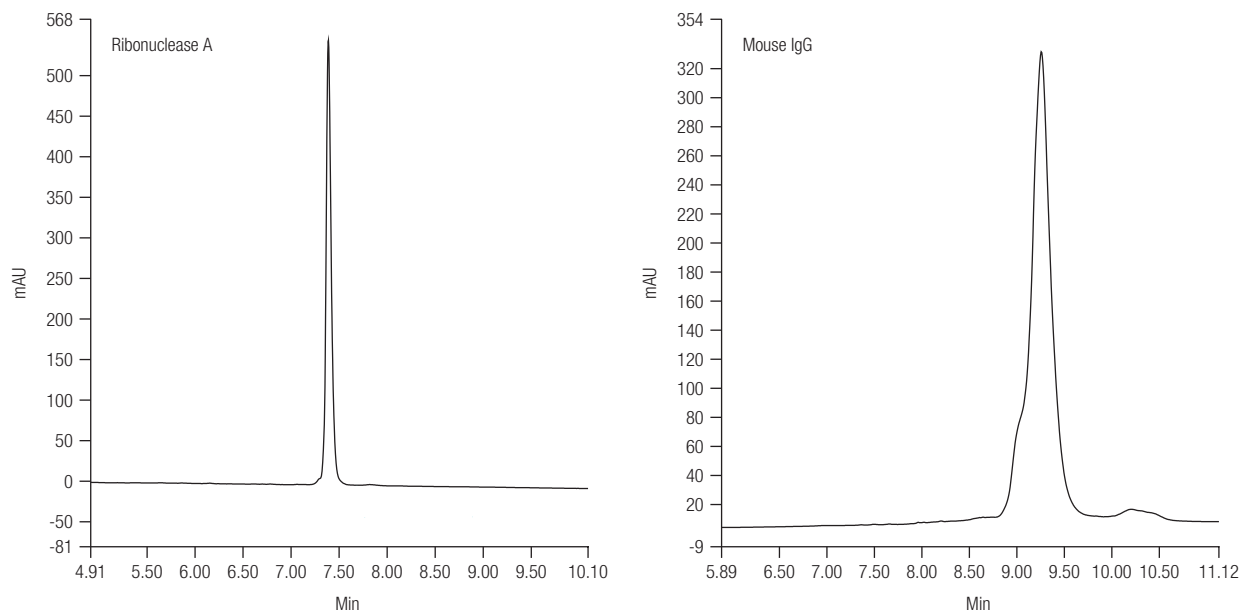


Figure 1. Chromatography of ribonuclease A and mouse IgG at 214 nm on a MabPac RP column.

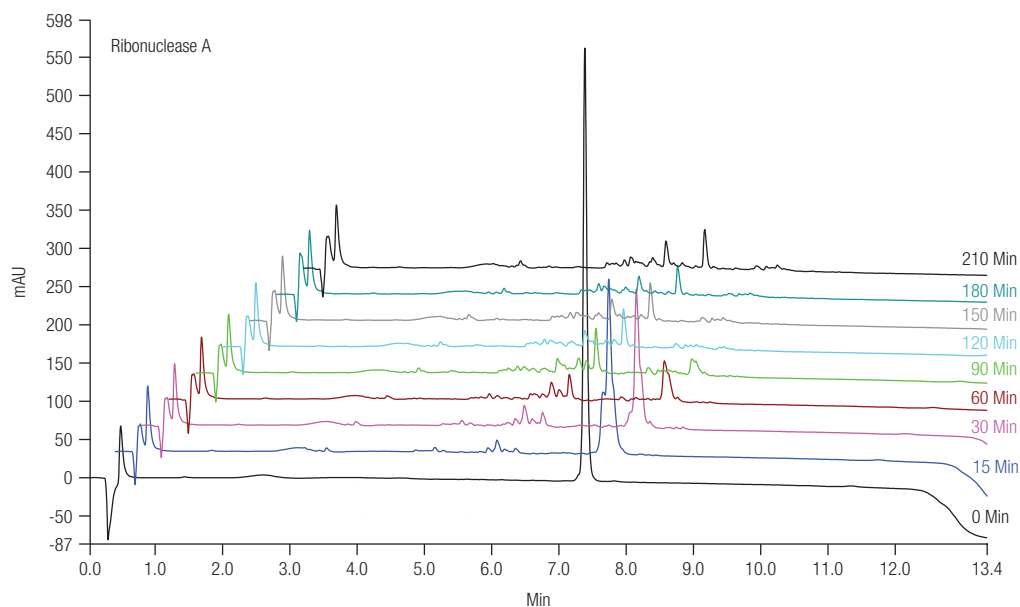


Figure 2. Chromatogram overlay of the digestion time points for ribonuclease A. Complete digestion is achieved in 120 minutes, when the peptide fingerprint is stable and the intact peak is no longer visible.

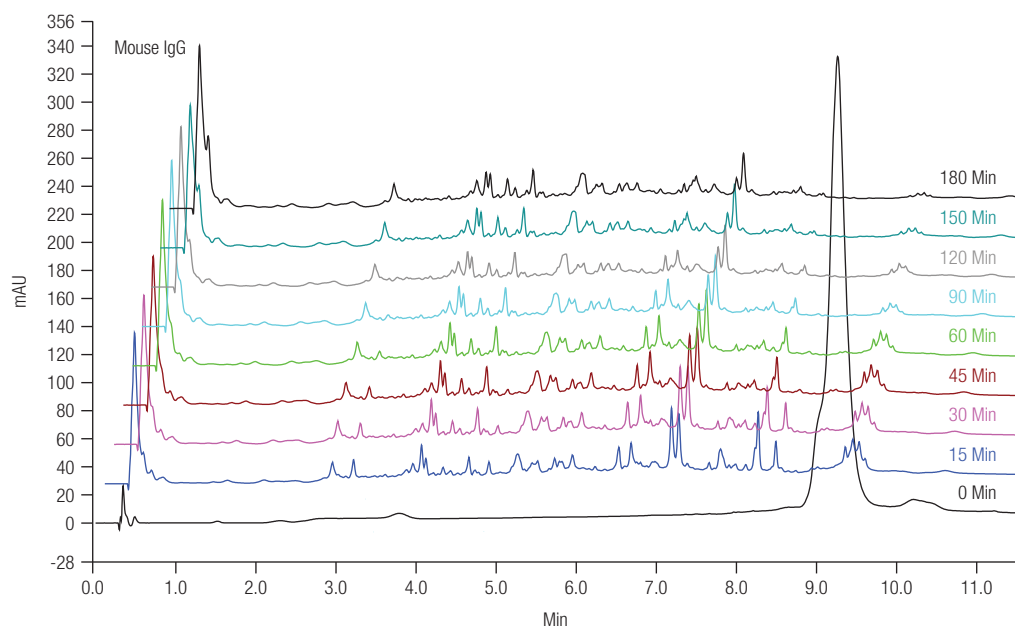


Figure 3. Chromatogram overlay of the digestion time points for mouse IgG. Initial digestion proceeds very fast and most of the sample is digested in the first 15 minutes. After 60 minutes the peptide fingerprint is stable and the intact peak is visible only in trace amounts.

Ribonuclease A is a small protein with a molecular weight of only 13.7 kDa. However, it is known to be very resilient to conventional tryptic digestion due to its heat stability and refolding ability.¹ Inspection of the chromatographic data shows that ribonuclease A, with the SMART Digest kit, is fully digested within 2 hours (Figure 2).

Compared to ribonuclease A, the digestion of mouse IgG proceeds much faster despite its considerably larger size. Most of the sample is digested in the first 15 minutes; after 60 minutes the peptide fingerprint is stable.

The unique features of the SMART Digest kit result in complete, fast digestions of normally challenging analytes. Table 3 lists the digestion times of a number of proteins previously tested. Even challenging samples such as thyroglobulin are digested in as little as 4 hours.

Table 3. Typical SMART digestion times of a series of protein.

Protein	SMART Digestion Time
Insulin	4
BSA	<5
Carbonic Anhydrase	<5
Lysozyme	<5
Apo-B	30
IgG in 50 μ L plasma	75
Thyroglobulin	240
C-reactive protein	240

Conclusion

- A simple method has been developed to identify the digestion endpoint with the SMART Digest kit.
- Complete SMART digestion method optimization is achieved in less than a day from start to finish.
- Ribonuclease, a protein known to be challenging to digest, is completely lysed within 2 hours.
- The method is applicable to any extracted protein sample.

Reference

1. Lang, K, Use of a trypsin-pulse method to study the refolding pathway of ribonuclease, *Eur. J. Biochem.*, 1986, 159, 275-281.

Useful Links

AppsLab Library

The eWorkflow and the Chromeleon Backup (cmbx) file can be downloaded at AppsLab Library:

www.thermofisher.com/appslib

www.thermofisher.com/SMARTdigest

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