Impact on Tryptic Digestion when Detergents and Chaotropes are Essential: A Case Study with Ribonuclease A

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Key Words

SMART Digest, 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, urea, CHAPS, RIPA, OGS, additives, detergents, chaotropes

Goal

To investigate the effect of four common additives on the digestion of ribonuclease A, a small, digestion-resistant, heat-stable protein, using the Thermo Scientific™ SMART Digest™ kit.

Introduction

Standard in-solution protocols for trypsin digestion are lengthy, multifaceted, and prone to irreproducibility. The SMART Digest kit eliminates these issues by providing a protocol that is:

- Highly reproducible

- Quick and easy to use

- Highly amenable to automation

The SMART Digest kit is shown to provide a fast, efficient, and reproducible tryptic digestion in a fraction of the time compared to a standard in solution digest method.

The SMART Digest buffer has been optimized to provide optimum digestion efficiency for most protein samples, without the need for addition of chaotropes or detergents. Occasionally, proteins may be resistant to digestion and require these additives. The main reasons for this include:

- Solubility – very large, hydrophobic proteins, such as membrane proteins, may be difficult to solubilize efficiently and thus require the addition of large quantities of detergents

- Heat stability/folding stability – proteins featuring a very stable secondary and tertiary structure require larger quantities of chaotropes to aid the unfolding process
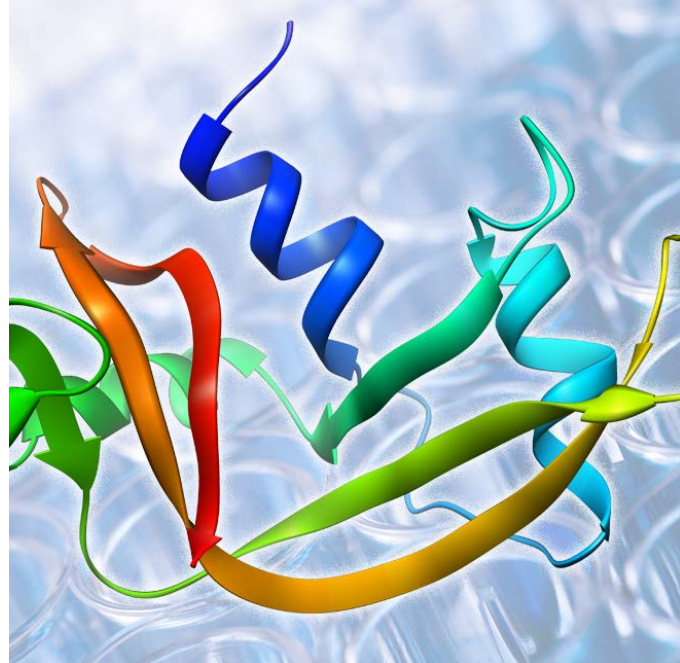
In this study we have investigated the effect of three commonly used detergents (RIPA, CHAPS, and OGS) and one common chaotrope (urea) on the digestion of ribonuclease A. This protein is particularly resistant to tryptic digestion due to its heat resistance and ability to re-fold to its native structure even when subjected to temperatures as high as 90 °C.[1] RIPA and urea were chosen as representative of additives commonly used in cell lysis.

Ribonuclease A was monitored during digestion with the SMART Digest kit, with and without the addition of each chaotrope in various concentrations. The rate of ribonuclease A depletion was compared under all conditions.

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Experimental

Digestion

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

Consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Fisher Scientific™ Optima™ acetonitrile (ACN) (P/N 10001334)
- Fisher Scientific trifluoroacetic acid (TFA) (P/N 10294110)
- RIPA Lysis and extraction buffer (P/N 89900)
- Urea, 99%, ACS Reagent (P/N 10462985)
- CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate) (P/N 28300)
- OGS (Octyl-beta glucoside) (P/N 28310)

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

Sample Handling

- 2 mL 96 well square plates (P/N 60180-P202)
- Thermo Scientific™ WebSeal™ 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 and lid is used to perform the digestion.

Sample Pretreatment

Ribonuclease A stock solution:

A stock solution of ribonuclease A from bovine pancreas was prepared in water at a concentration of 2 mg/mL. The solution was sonicated to ensure complete dissolution of the protein.

SMART Digest buffer + urea:

All urea solutions in SMART Digest buffer were prepared at a concentration 25% higher than that required for the digestion study. This was applied in order to take into account for a 25% dilution factor applied when the ribonuclease A stock solution was added to the SMART Digest buffer prior to digestion.

A 5.33 M stock solution of urea in SMART Digest buffer was prepared and subsequently diluted in SMART Digest buffer according to Table 1.

Table 1. Dilution series for the preparation of urea in SMART Digest buffer.

Solution	Concentration of Urea (Before Digestion Start)	Concentration of Urea (Digestion Reaction)	Dilution Protocol
A	0.67 M	0.5 M	250 µL stock solution + 1.750 mL SMART Digest buffer
B	0.27 M	0.2 M	50 µL stock solution + 950 µL SMART Digest buffer
C	0.133 M	0.1 M	200 µL solution A + 800 µL SMART Digest buffer
D	0.067 M	0.05 M	100 µL solution A + 900 µL SMART Digest buffer

SMART Digest buffer + RIPA/CHAPS/OGS

Solutions of each detergent were prepared from 10% w/v stock solutions, as described in Table 2.

Table 2. Preparation of detergent solutions.

Solution	Concentration of Detergent for Digestion Reaction (v/v)	Preparation for 1 mL (10 mL for 0.01% stock)
A	0.50%	5 µL stock + 995 µL SMART Digest buffer
B	0.20%	2 µL stock + 998 µL SMART Digest buffer
C	0.10%	1 µL stock + 999 µL SMART Digest buffer
D	0.05%	1 µL stock + 1.999 mL SMART Digest buffer
E	0.01%	100 µL solution C + 900 µL SMART Digest buffer

Sample Preparation

Ribonuclease A digestion

First, 75 µL of SMART Digest buffer, with or without additive, were pipetted into each SMART Digest trypsin tube. Then, 25 µL of the 2 mg/mL ribonuclease A was added to each tube (50 µg final protein amount). To ensure a uniform starting time 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, and 150 minutes for analysis.

For all sample sets, digestion was stopped by simply passing the sample through the filter plate using positive pressure. This removed the sample from the immobilized trypsin beads. The eluent was subsequently analyzed using LC-UV.

Standards corresponding to t=0 minutes, containing the maximum ribonuclease A concentration, were prepared by diluting 25 µL of ribonuclease A solutions with 75 µL of SMART Digest buffer with the corresponding additive.

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:	Thermo Scientific™ MAbPac™ RP, 50 × 2.1 mm (P/N 088648)
Mobile Phase A:	Water + 0.1 % TFA
Mobile Phase B:	Acetonitrile + 0.08% TFA
Gradient:	See Table 3
Flow Rate:	0.5 mL/min
Column Temp.:	80 °C
Autosampler Settings:	The autosampler settings were optimized for the WPS 3000 TRS (see Table 4)
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 3. LC gradient conditions.

Time (min)	%A	%B
0	100	0
1	100	0
11	50	50
11.01	0	100
15	0	100
15.01	100	0
20	100	0

Table 4. Autosampler settings.

	SMART Digest Buffer SMART Digest Buffer + Urea SMART Digest Buffer + RIPA	SMART Digest Buffer + CHAPS	SMART Digest Buffer + OGS
Injection Volume (µL)	5	5	5
Draw Speed (µL/s)	0.05	3	1
Draw Delay (s)	3.0	3	3
Dispense Speed (µL/s)	10.0	20	20
Dispense Delay (s)	2.0	2	2
Dispense to Waste Speed (µL/s)	1.0	20	20
Sample Height (mm)	2.0	2	2

Data Processing

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

Results and Discussion

Figure 1 shows the progress of the ribonuclease A digestion with no additives at different time points. Digestion is complete at 120 minutes. This can be determined as the intact protein peak is no longer visible and the tryptic peptides are consistent.
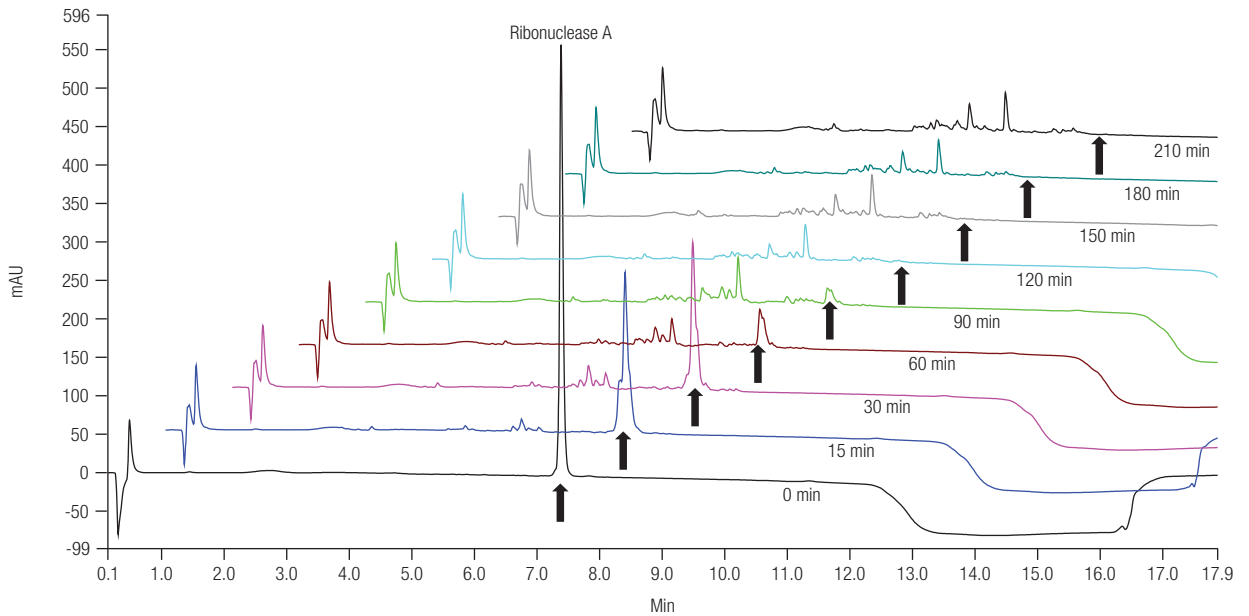

Figure 1. Chromatogram overlay of the digestion time points for ribonuclease A (no additive).

The peak area of ribonuclease A was measured for each time point in each condition studied. To compare digestion progress with each additive, the rate of decrease of ribonuclease A peak area was monitored. For simplicity, each condition was monitored against digestion in SMART Digest buffer without additives.

SMART Digest Buffer + Urea

Figure 2 shows the peak area decrease of ribonuclease A with the addition of urea. For simplicity, all areas have been normalized with respect to the peak area at t=0 in the same condition.

It is evident that the addition of urea in this case results in a small reduction in the digestion rate in the first 60 minutes, but has no significant impact on the overall digestion completion time.
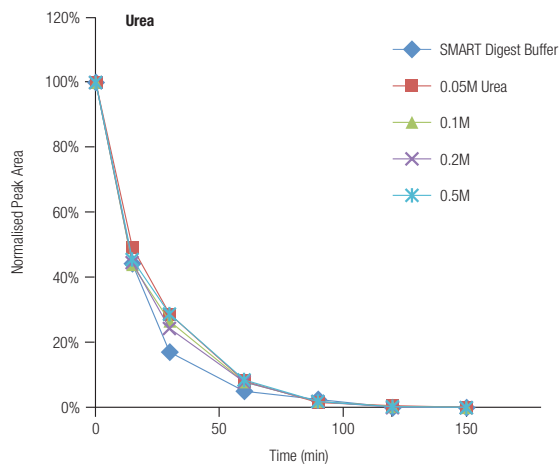
Urea is commonly used in very high concentrations (4–8 M) to efficiently lyse cell cultures for subsequent digestion. A concern in these cases is that in excessive concentrations, urea will readily precipitate once heated to 70 °C, as required for tryptic digestion with the SMART Digest kit. The results above indicate that a simple dilution of the sample to a concentration of 0.5 M urea or below can be applied and prevent this.

SMART Digest Buffer + RIPA

Figure 3 shows the area depletion of ribonuclease A with the addition of RIPA. For simplicity, all areas have been normalized with respect to the peak area at t=0 in their the same condition.

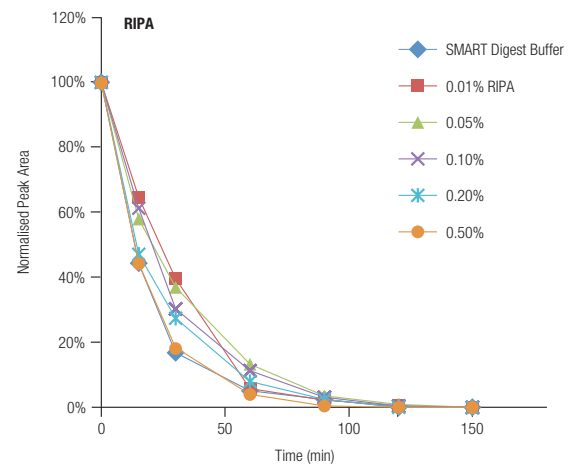Figure 2. Area depletion of ribonuclease A with the addition of urea in concentrations up to 0.5 M.

Figure 3. Area depletion of ribonuclease A with the addition of RIPA up to 0.5% w/v.

The effect of RIPA on the digestion of ribonuclease A is largely concentration dependent. A significant reduction of up to 20% in digestion rate is observed in the first hour for concentrations up to 0.1%. However, beyond this concentration the rate is comparable to digestion in SMART Digest buffer without additives. A possible explanation for this is that beyond a concentration of 0.1% RIPA, the additives present in this buffer provide an additional denaturing effect on ribonuclease A, which favor the digestion to a greater extent than any partial trypsin inhibition.

SMART Digest Buffer + CHAPS

The effect of CHAPS on the digestion of ribonuclease A was found to be uniformly negative, with a reduction in digestion rate of up to 32% (Figure 4). Unlike RIPA, this trend is not concentration-dependent.

SMART Digest Buffer + OGS

The effect of adding OGS to the digestion of ribonuclease A was found to have a mild positive effect on the digestion rate. In the first 15 minutes of digestion, OGS showed a 10–20% increase in ribonuclease A depletion (Figure 5). The effect is particularly pronounced at smaller concentrations of OGS (up to 0.1%).
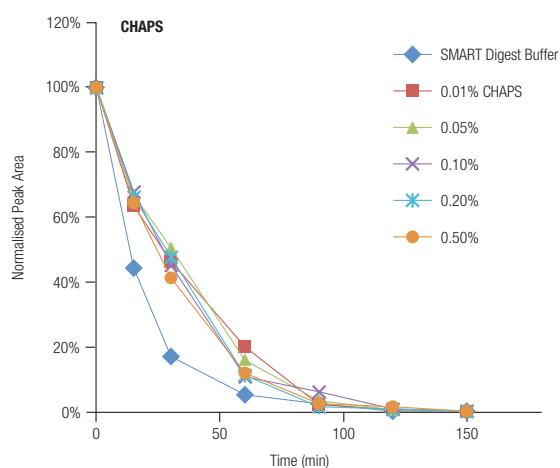
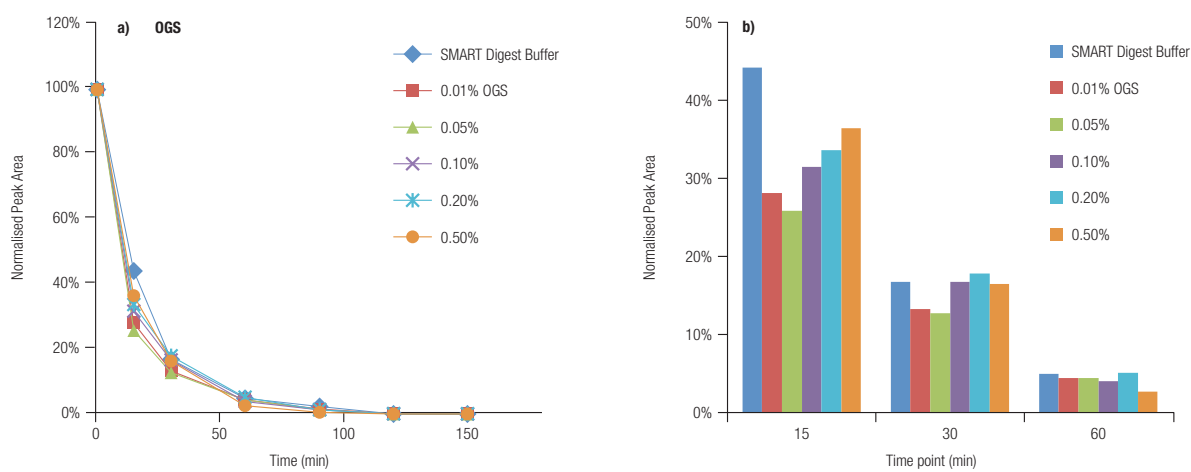Figure 4. Area depletion of ribonuclease A with the addition of CHAPS up to 0.5% w/v.

Figure 5. a) Area depletion of ribonuclease A with the addition of OGS up to 0.5% w/v. b) Individual time point bar chart view for time points up to 60 minutes.

To verify if the digestion rate increase was statistically significant, the time points were repeated in triplicate; for simplicity only three concentrations were studied (SMART Digest buffer, 0.1% and 0.5% OGS). Figure 6 shows the measured digestion time points for the triplicate experiments. The time curve did not show an obvious rate increase (Figure 6a); however, the detailed view of the 15 minute time point shows a statistically significant increase in digestion of approximately 7% (Figure 6c). At 30 minutes an apparent increase of approximately 5% was observed, but this was found to be within the measured error and therefore not significant.
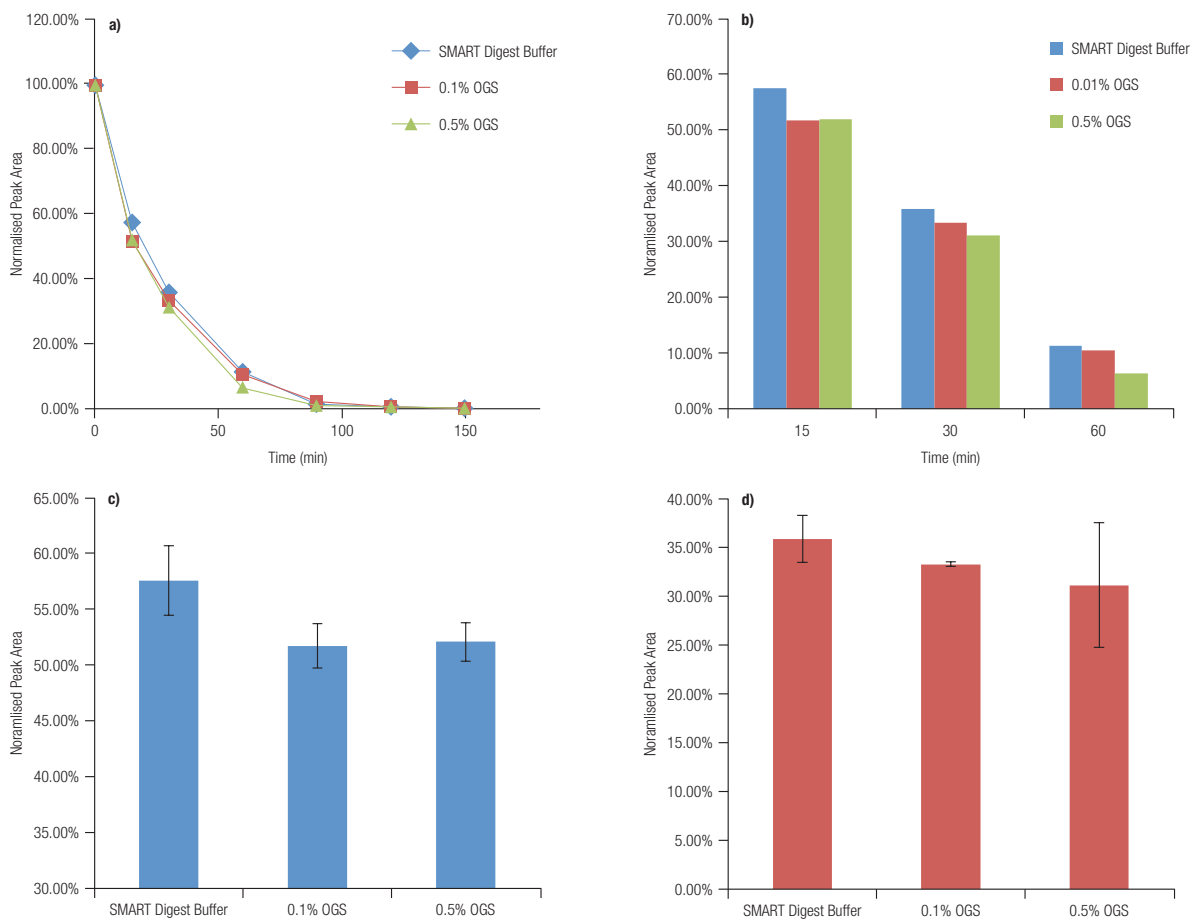
Figure 6. a) Average area depletion for n=3 of ribonuclease A with the addition of OGS (0, 0.1% and 0.5%)

b) Individual time point bar chart view for time points up to 60 minutes

c) Detailed view of 15 minutes, including %RSD above and below the average

d) Detailed view of 30 minutes, including %RSD above and below the average

In cases where troublesome analytes are to be digested, the addition of OGS forms a particularly promising prospect. The additive does not inhibit digestion and can lead to an improved digestion rate.

Conclusion

Using a case study with ribonuclease A, we have shown that:

- Addition of up to 0.5 M urea does not significantly impact the digestion rate.

- In cases where high urea amounts are usually used for cell lysis, a reduction in concentration to 0.5 M prevents precipitation.

- The addition of OGS has a slight rate enhancement effect.

- The addition of RIPA, for ribonuclease A digestion, results in a concentration-dependent effect, where initial enzyme inhibition is overcome by improved substrate solubilization at higher concentrations only.

- The addition of CHAPS as a detergent leads to an overall decrease in ribonuclease A digestion rate.

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/appslab

www.thermofisher.com/SMARTdigest

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