Application Note 21179

Selecting Buffers to Remove Uncertainty in Tryptic Digestion

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Key Words

Peptides, proteins, monoclonal antibody, mAb, SMART Digest, protein digestions, Accucore C18, trypsin digestion, buffers, biomolecules, biotherapeutics, biopharmaceutical

Goal

To demonstrate how Thermo Scientific™ SMART Digest™ kits remove uncertainty associated with conventional solution-based tryptic digestion protocols, resulting in higher reproducibility and sample characterization.

Introduction

Protein digestion is a fundamental technique employed in biopharmaceutical and proteomic applications. It is used to analyze the sample and identify structural features or post-translational modifications (PTMs). Despite its widespread use, protein digestion still provides many analytical challenges. The optimum 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 do not affect trypsin itself
- Use the minimum amount of trypsin to protein to prevent partial digestion

Since optimization of all steps listed above is required, the process can be lengthy and complex and often leads to poor reproducibility.

The SMART Digest kit mitigates lengthy and complex method development by providing a protocol with the following attributes:

- Highly reproducible
- Quicker and easier to use
- Requires no detergent
- Temperature-induced denaturing of proteins through the use of a heat-stable trypsin
- Reduced autolysis through the use of immobilized trypsin
- Results in fewer chemically-induced PTMs

Trypsin digestion of insulin using the SMART Digest kit and a series of commonly used buffer species and additives were compared. In order to determine the impact of various buffers and additives on the trypsin digestion, a partial digestion of insulin was performed.

Insulin provides an ideal candidate for this study, as it creates a very simple peptide map. The partial digestion was used to assess variance of enzyme activity. When exposed to trypsin, human insulin forms two major products. There is a C-terminal peptide sequence and a larger N-terminal sequence (Figure 1). These two peaks are easily resolved from the intact protein using reversed phase chromatography (Figure 2).

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Protein digestions are often performed in the presence of chaotropes, surfactants, salts, and organic solvents. Due to the general robustness of trypsin, it is often assumed that activity is negligibly affected by these additives. In this study, the activity of trypsin from the SMART Digest kit was assessed by varying the digestion buffer conditions and comparing these to the SMART Digest buffer. The results show that trypsin activity varies significantly depending on the buffer used. Additives such as guanidine HCl and urea have a concentration-dependent adverse effect on the digest efficiency. Furthermore, buffer species such as phosphate buffer saline (PBS) and ammonium bicarbonate (ABC) were also found to adversely affect digestion.

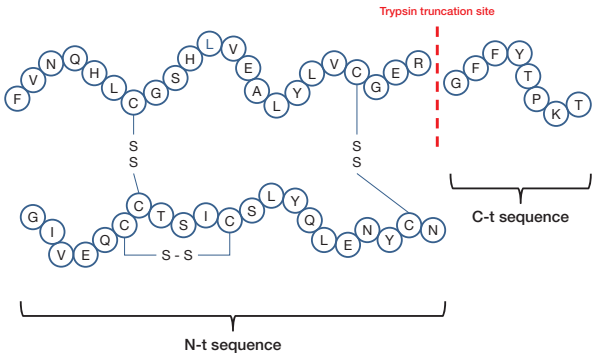


Figure 1. The amino acid sequence of insulin.

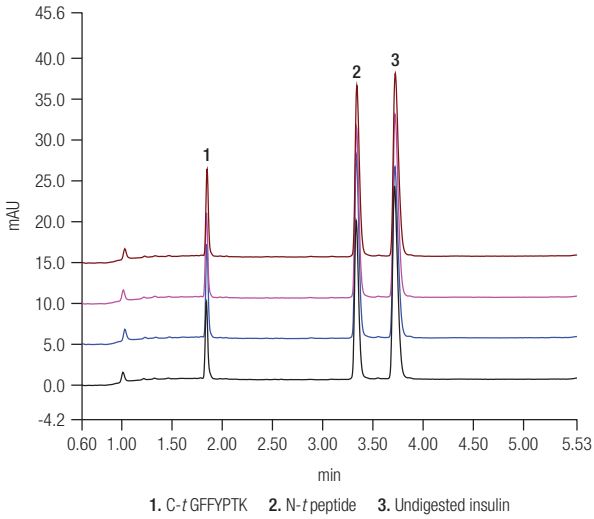


Figure 2. Typical chromatogram obtained for the LC/UV analysis of insulin partial digest.

Experimental

Digestion

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

Sample Handling

- 96-well collection plate provided in SMART Digest kit

Column

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

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 2,2,2-trifluoroethanol (TFE) (P/N 10468733)
- Fisher BioReagents formamide (P/N 10440464)
- Thermo Scientific Pierce guanidine HCl (P/N 11821365)
- Fisher BioReagents™ Tween™ 20 (P/N 10113103)
- Pierce octyl-beta-glucoside (P/N 28310)
- Fisher BioReagents deoxycholate (P/N 10346653)
- Fisher Scientific analytical grade trifluoroacetic acid (P/N 10112740)
- USP-grade insulin was purchased from a reputable supplier.

Sample Handling Equipment

Heater/shaker equipped with PCR block and heated lid.

Digestion Protocol

As listed in Table 1, 100 µg/mL solutions of insulin were prepared in a variety of buffers.
Experiments were designed to compare the performance of the SMART Digest buffer against six variables commonly used in solution based digests. The variables studied are also listed in Table 1.

Table 1. Buffer systems used to prepare insulin solutions.

Buffer System Studied		Variable Studied
SMART Digest buffer		n/a
PBS ABC Tris (0.05–0.5 M) HEPES		Buffering ion
4.50	NaCl (0.1–2 M)	Electrolyte
Tris + 10 mM $CaCl_2$ +	pH 7–9	pH
	Gdn HCl (0.5–6 M) Urea (0.5–8 M) OGS (0.05–0.2%)	Chaotropes
	Tween (0.01–0.1% v/v) Zwitt (0.1–1%) SDC (0.1–1%)	Detergents
	DMSO (5–20% v/v) ACN (5–20% v/v) MeOH (5–20% v/v) IPA (5–20% v/v) Formamide (5–20% v/v) TFE (5–20% v/v)	Co-solvents

Each insulin solution was subsequently digested by adding 200 µL of the solution to the SMART Digest immobilized trypsin tube. The mixture was incubated with agitation at 70 °C for 1 minute. This provided a partial digestion.

The peak area of the C-t sequence was measured in all buffer systems for comparison. Following incubation, the digestion reaction was quenched by means of acidification.

Separation Conditions

Insulin digestion products were analyzed by LC/UV using the gradient outlined in Table 2. Separation was achieved on an Accucore C18 column, 2.6 µm particle, 50 × 2.1 mm, as shown in Figure 2.

Instrumentation	Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC System
Column	Accucore C18 (50 × 2.1 mm, 2.6 µm particle) (P/N 7126-052130)
Mobile phase A	2% acetonitrile, 98% water, 0.1% TFA
Mobile phase B	90% acetonitrile, 10% water, 0.1% TFA
Flow rate	0.5 mL/min
Column temp.	40 °C
Injection details	25 µL

Table 2. LC gradient conditions.

Time (min)	% A	% B
0.01	90	10
1	75	25
6	50	50
6.01	10	90
7.5	10	90
7.51	90	10
9	90	10

Software

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

Results and Discussion

The various additives and their concentrations had a dramatic effect on the digestion efficiency of the SMART Digest trypsin. Figure 3 summarizes the results found.

A small number of additives showed minor impact. Among the buffering ion screening (Figure 3a), HEPES and Tris up to 0.1 M gave a less than 20% reduction in tryptic activity. Equally, the chaotrope octylglucoside (Figure 3d) showed to have only a small impact on the digestion efficiency; the effect was not found to be concentration-dependent.

The buffering ion, however, was found to be crucial for optimal activity. Ammonium bicarbonate (ABC) and phosphate buffer saline (PBS) showed a much reduced rate of digestion, giving a 90% and 70% product reduction, respectively (Figure 3a).

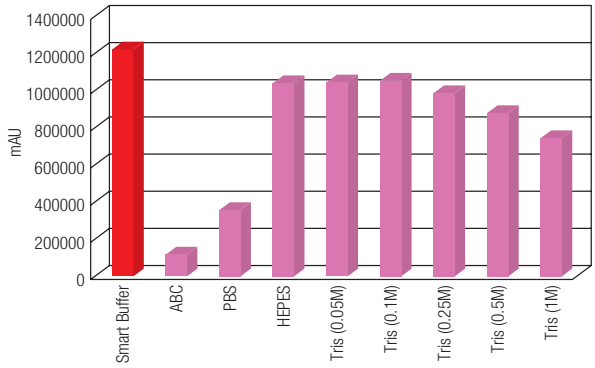
When the pH of the buffer was scrutinized (Figure 3c), tris buffer at pH 8 showed comparable performance to the results obtained using the SMART Digest kit. However, the higher pH is known to be a likely cause of chemically-induced PTMs and, therefore, deviations in the SMART Digest buffer pH away from pH 7.4 were not made in order to prevent this effect.

Addition of sodium chloride was found to give activity with a uniform decrease of 20–30%, regardless of the concentration studied (Figure 3b).

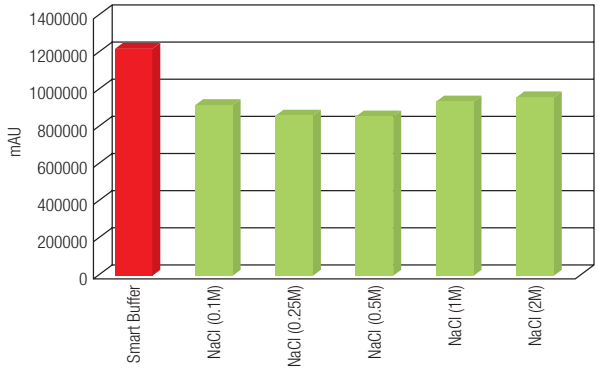
The addition of detergents in solution-based tryptic digests is common and serves the purpose of aiding protein solubility. The quantity of detergent, however, is crucial in being able to subsequently extract the triptic peptides. The most pronounced negative effect was observed with the addition of zwitterionic detergent (Zwitt) and sodium deoxycholate (SDC) (Figure 3e). In both cases, minimal quantities resulted in almost complete absence of digestion product.

Digestion in the presence of guanidine hydrochloride (Gdn HCl) or urea (both Figure 3d) was found to be negatively affected, with a pronounced concentration-dependent effect. At concentrations above 2M, these additives were both found to result in almost no digestion product. A similar trend was observed when organic co-solvents were studied (Figure 3f).

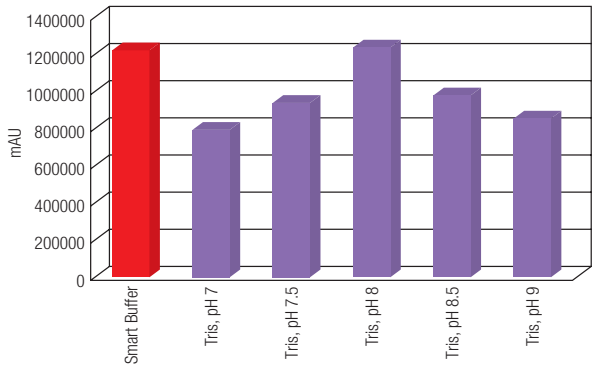
A Effect of buffering ion



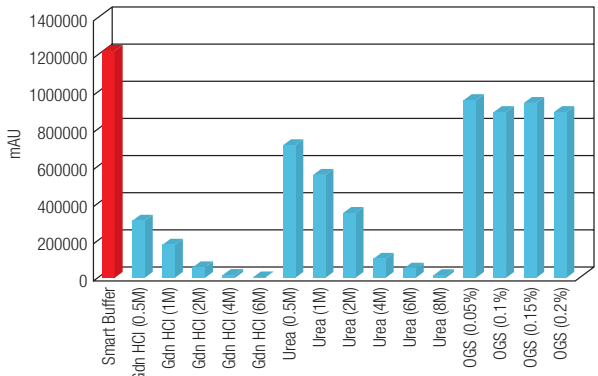
B Effect of salt concentration



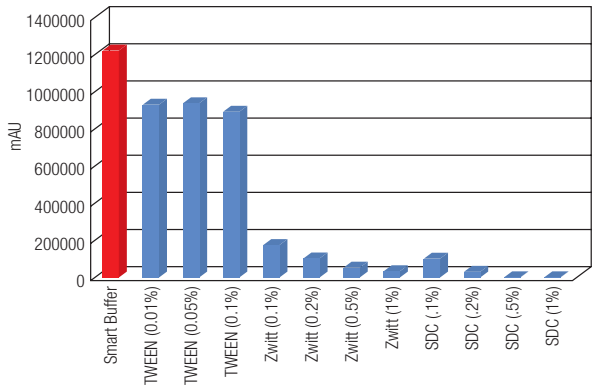
C Effect pf pH



D Effect of chaotropes



E Effect of detergents



F Effect of co-solvents

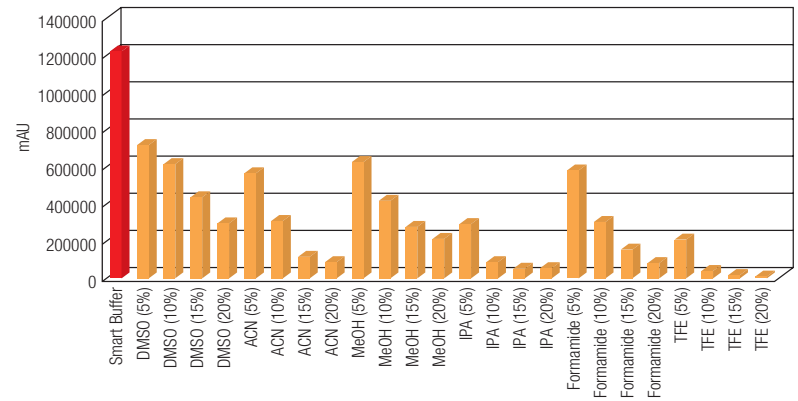


Figure 3. Peak areas of C-t peptide (GFFYTPKT) generated from the digestion of the protein using the SMART Digest kit as a function of a) variation of buffering ion; b) salt concentration; c) pH; d) chaotropes; e) detergents; and f) organic co-solvents. The SMART Digest buffer result is shown as a red bar in each figure.

Conclusion

The SMART Digest kit outperforms conventional solution-based tryptic digestion buffer systems, while providing a fast, simple, and clean method. Fewer steps are required to achieve complete digestion of samples.

In this application note, trypsin digestion of insulin was compared to the SMART Digest buffer and a series of commonly used buffer species and additives. From the results it is obvious that the following are true:

- Digestion efficiency is highly dependent on the buffer and additives used.
- The SMART Digest buffer outperforms all 6 variables compared.

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