Overview

Purpose: Develop a synthetic protein (concatenated peptide) that can be used to standardize and monitor proteolytic digestion prior to selected reaction monitoring (SRM) assays or other mass spectrometry (MS) assays.

Methods: A long, concatenated peptide was created with multiple cleavage sites varying in accessibility as determined by single or multiple arginines and lysines.

Results: The data demonstrate that the accessibility of the digestion sites (as determined by single or multiple arginines or lysines) had a significant effect on the optimal incubation times for digestion. Therefore, the concatenated peptide can be used as a quality control (QC) tool for monitoring digestion efficiency in experimental samples before quantitative SRM (and other MS-based) assays.

Introduction

In many proteomic applications, proteins are subjected to proteolytic digestion. Following digestion, peptides are often analyzed by mass spectrometry resulting in qualitative or quantitative data. Although enzymatic protein digestion is broadly used, the results may be highly variable depending on sample, enzyme-to-protein ratio and digestion conditions. Because enzymatic digestion is the starting point for many proteomic experiments and assays, inconsistent sample preparation can compromise the downstream analysis of the resulting data, especially in SRM assays where accurate quantification of peptides is crucial. Therefore, a thorough understanding of proteolytic digestion efficiency during the sample preparation process is critical to the success of these methods.

Methods

Samples

Human serum samples were collected from a donor with full consent and approval.

Synthetic Peptides

A concatenated synthetic protein which could be used to assess the quality and efficiency of proteolytic digestion during sample preparation was developed. The 4707 Da protein (see Figure 1) contained 42 amino acids, eight of which were heavy isoforme labeled (either arginine or lysine). The protein was purified by HPLC and 98% pure and stored frozen in solution at a 5 pmol concentration. Following complete proteolytic digestion, the synthetic protein produced exactly four proteolytic peptides. In addition, light and heavy isoforme-labeled versions of the resulting proteolytic peptides were synthesized. The proteolytic QC protein was tested by adding a known concentration to a serum sample and then subjecting the mixture to enzymatic digestion by mass spectrometry analysis in a triple quadrupole SRM-based assay.

Proteolytic Digestion

Proteolytic digestion was carried out as previously described (1, 2) except three different buffer compositions (Table 1) were tested, and aliquots were taken at time points (1, 4, 24 hr) for SRM assays. SRM Assay Development SRM assays were developed as previously described (1, 2), and as shown in Figure 1B.

Results

Serum samples containing the synthetic protein Figure 1A, were subjected to enzymatic digestion. In order to understand the efficiency of proteolysis, each sample underwent a time course experiment in three different buffers, as shown in Table 1. Aliquots of each sample were taken at the following time points during digestion: 1 hr, 4 hr and 24 hr. At each time point, an aliquot of sample was removed for triplicate analysis. Calibration curves were generated using light and heavy isoforme-labeled versions of the peptides from the proteolytic protein and the LLOD and LLOD in serum matrix were calculated. The LLOD of the synthetic peptides ranged from 500 attomoles to 1 femtomole (data not shown). The addition of the proteolytic QC protein to the SRM assay experiments provided increased confidence in the quantitative measurements for targeted proteins in the experimental sample.

Conclusion

The concatenated synthetic protein provided a simple, straightforward way to measure the efficiency of proteolytic digestion prior to SRM assays.

Longer incubation times were required to produce cleavage of “difficult” sites such as those with multiple arginines or lysines.

However, longer incubation times reduced recovery of peptides bound by “easy” sites (single arginines or lysines).

Four hour incubation times are sufficient to provide cleavage at all sites with an overall efficiency of 60-80% and minimize peptide losses.

Buffer composition is important to ensure optimum digestion efficiency. Buffer containing SDS with subsequent SDS removal reduced efficiency dramatically.

References
