Improving the Efficiency and Throughput of an Enzymatic Digestion of Klotho using Pressure Cycling Technology (PCT)

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Overview

Purpose: To improve the efficiency and throughput of a tryptic digestion of Klotho, a highly gycosylated membrane protein.

Methods: Recombinant Mouse Klotho (R&D Sciences 1819-KL-050) was reduced, alkylated and digested with trypsin using various methods, including Pressure Cycling Technology (PCT). Heavy isotope labeled internal standards allowed for the determination of absolute quantitation and digestion efficiency, using LC-SRM on a TSQ Vantage. Samples were also run on the Exactive mass spectrometer in order to determine how non-targeted peptides behaved with different digestion methods.

Results: PCT can significantly improve the efficiency and throughput of a tryptic digestion when monitoring the FSISWAR peptide of Recombinant Mouse Klotho.

Introduction

Klotho is a transmembrane protein that is thought to be involved with insulin regulation and aging. Like many transmembrane proteins, Klotho is difficult to fully digest with trypsin using standard methods. A complete enzymatic digestion prior to the quantitation of peptides with LC-MS would be highly desirable in order to obtain maximum assay sensitivity. In this work, Pressure Cycling Technology (PCT) is shown to provide significant improvement in both the efficiency and throughput of an enzymatic digestion of Klotho, when monitoring specific peptides. Samples were analyzed with both LC-SRM on a TSQ Vantage triple quadrupole MS and LC-Full Scan Accurate Mass on an Exactive MS.

Methods

All samples were aliquoted into 96 well plates post digestion and evaporated to dryness. Each sample was then brought up in 30 µL of a 200 µg/mL solution of glucagon in 97% water and 3% acetonitrile with 0.2% formic acid, including all isotopically labeled internal standards. The glucagon solution significantly reduces the adsorption of peptides to plastic.

LC: Accela Pump and Thermo Pal
Column: 150x1 3um Hypersil Gold Heated to 50 Degrees C
Solvent A: Water 0.2% FA
Solvent B: ACN 0.2% FA
Gradient/Flow Rate: A 13 minute gradient from 5% to 45% B was utilized. Flow rates during peptide elution were set at 180uL/min.
MS: TSQ Vantage, Exactive

Figure 1 (left) displays the TIC for the LC-SRM method on the TSQ Vantage. Peak widths for all 8 peptides monitored were 12 seconds or less (Figure 1 Right). 5 transitions were monitored for each peptide. At least 12 scans were acquired across each peak for each transition. Total method run time was 22 minutes.

In order to determine digestion efficiency, the following formula was utilized:

(Amount of Endogenous Peptide Measured /Moles of Recombinant Added)*100

Where the absolute amount of endogenous peptide was determined by single point relative quantitation using spiked in heavy internal standards.

FIGURE 1. LC-SRM with the TSQ Vantage

Standard Trypsin Digestion

Initially, 4 standard trypsin digestion methods were tested on recombinant mouse Klotho. These included the denaturing and reducing starting buffers listed below:

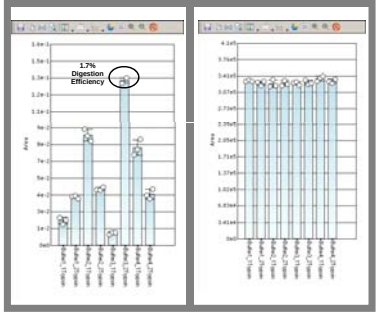
Buffer 1- 8M GuHCl/150mM Tris/10mM DTT pH 8.5
Buffer 2- 8M Urea/2.5% n-propanol/200mM Tris-HCl/10mM DTT pH 8.5
Buffer 3- 250mM Ammonium Bicarbonate/50%MeOH/10mMDTT pH 8.5
Buffer 4- 8M Urea/2.5% n-propanol/200mM Tris-HCl/10mM DTT pH 8.5/0.01% Protease Max

Following denaturing and reduction, all samples were alkylated, diluted and digested with trypsin overnight (1:50 – trypsin:protein). A second addition of trypsin, with a 24 hour incubation was also included. All samples were then put through SPE, dried down and brought up in a solution of heavy isotope labeled peptides before running with LC-SRM on the TSQ Vantage.

From the 8 tryptic peptides monitored, the FSISWAR peptide corresponding to amino acids 151-157 of Klotho, provided the best performance. The 50% MeOH digestion conditions (Buffer 3) with a second addition of trypsin gave a digestion efficiency of 1.7%, highest of all the conditions.

Figure 2 (left) displays relative quantitation of peptide FSISWAR, normalized to its heavy isotope labeled internal standard, for the various standard digestion methods used. Figure 2 (right) depicts the peak area values for the heavy internal standard across all runs, with CV's of less than 3%.

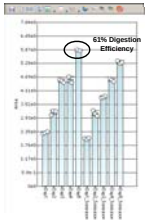
FIGURE 2. FSISWAR Peptide and Various Digestion Methods



Optimizing the Standard Trypsin Digestion

After determining the best digestion conditions for FSISWAR, experiments were developed to improve the 1.7% digestion efficiency obtained. The 50% MeOH denaturing and reduction conditions (Figure 2, Buffer 3) showed significant improvement with a second addition of trypsin. As a result, 5 continuous trypsin additions over the course of 5 days were run using Buffer 3. These experiments were run with and without sonication during the denaturing and reduction step. In addition, the post digestion SPE step was removed and replaced by an online desalting utilizing the divert valve.

FIGURE 3. Optimizing Standard Digestion Conditions for FSISWAR



Digestion Efficiency

Day1: 28%
Day2: 37%
Day3: 50%
Day4: 50%
Day5: 61%

Day1(Sonication): 25%
Day2(Sonication): 36%
Day3(Sonication): 43%
Day4(Sonication): 50%
Day5(Sonication): 57%

Figure 3 displays the results of optimizing the standard digestion conditions using Buffer 3 and the FSISWAR peptide, normalized against its heavy isotope labeled internal standard. Results show that removing the SPE step post-digestion improved efficiency by over 10 fold. In addition, subsequent daily trypsin additions increased digestion efficiency by approximately 10%. This suggests that in order to completely digest Klotho with the optimal standard method utilizing Buffer 3 and peptide FSISWAR, there would need to be 9-10 days of iterative trypsin additions. In this case, sonication showed no significant effect on digestion efficiency

Pressure Cycling Technology (PCT)

FIGURE 4. Picture of Barocycler

The Pressure Cycling Technology Sample Preparation System ("PCT SPS", Pressure BioSciences, inc.) employs rapid cycles of hydrostatic pressure to control biomolecular interactions. The hydrostatic pressure has been shown to accelerate certain enzymatic reactions, such as protein digestion with trypsin and other proteolytic enzymes, to prepare samples for analysis by mass spectrometry by promoting hydration of hydrophobic protein residues and altering substrate protein conformation, rendering it more susceptible for enzymatic digestion (1,2). The PCT SPS is comprised of a Barocycler (a semi-automated bench top instrument used to generate high hydrostatic pressure) and inert fluoropolymer sample containers, PCT MicroTubes. Depending on the instrument model, 12 or 48 samples may be processed simultaneously in this temperature-controlled high pressure system.

PCT Assisted Trypsin Disgestion

FIGURE 5. PCT Digestion Conditions

	20,000 psi, 55°C (2 hrs)	20,000 psi, 37°C (2 hrs)	35,000 psi, 55°C (2 hrs)	35,000 psi, 37°C (2 hrs)	37°C control digest (48 hrs)
50mM ambic/1mM CaCl2	X		X		
50mM ambic/1mM CaCl2 with 4% HFIP	X				
50mM ambic/1mM CaCl2 with 10% n-propanol	(X)		X		
50mM ambic/1mM CaCl2 with 10% MeOH	X		X		
50mM ambic/1mM CaCl2 with 20% MeOH	X				
50mM ambic/1mM CaCl2 with 25mM GuHCl	X				
50mM ambic/1mM CaCl2 with 0.4M urea		X		X	(X)

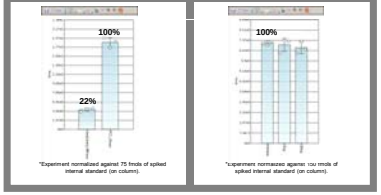
*Best 2 hour PCT Conditions and 48 hour Urea control digest are circled

PCT digestion conditions are shown in Figure 5 and described in detail below.

Denature, reduce and alkylate 30ug of Recombinant Mouse Klotho: Dilute protein in 50mM ambic with 0.01% AALS II (Protea) to 200µl. Vortex for 10 seconds. Add 2ul 1M DTT to 10 mM final. Incubate at 55 deg for 30 mins. Cool to room temp. Add 10ul of 1M IAA to 50 mM final concentration.Keep in dark. Alkylate at room temp for 60 mins. Add 90ul 50mM ambic to bring protein to 300ul (1ug/10ul). For each reaction, dilute 10ul reduced/alkylated protein (1ug) with 90ul appropriate digestion buffer. Then add 1ul trypsin (0.1ug, 1:10 enzyme-to-substrate ratio), cap the tubes, vortex briefly and incubate as described below, using the various conditions shown in Figure 5 .

PCT: 60 cycles (50 seconds at high pressure, 5 seconds at atmosphere). Remove MicroTubes and vortex. Open tubes and add another 1ul (0.1ug) of trypsin solution per sample. Vortex. Repeat PCT as above for another 60 cycles. Stop reaction by adding 10ul 50% formic acid (5% final conc). Total digestion reaction time was 2 hours.

FIGURE 6. PCT Digestion Efficiency Results using FSISWAR



*Experiment normalized against 75 fmole of spiked internal standard (on column).

*Experiment normalized against 100 fmole of spiked internal standard (on column).

Figure 6 (left) is a comparison of the best 2 hour PCT digestion conditions and a standard 48 hour urea control digest with neat recombinant mouse Klotho, both with 2 additions of trypsin (conditions circled in Figure 5). The 2 hour PCT conditions give 100% digestion efficiency for peptide FSISWAR and 22% for the 48 hour urea control.

Figure 6 (right) displays the results of the best 2 hour PCT digestion conditions for Klotho when used on samples with various amounts of plasma background, while measuring the FSISWAR peptide. Samples with spiked recombinant mouse Klotho and no matrix background were repeated and shown to reach 100% digestion efficiency as expected. In addition, samples with 26 and 260ug of plasma matrix per MicroTube and spiked recombinant mouse Klotho, were also shown to be near 100% digestion efficiency.

FIGURE 7. Other Peptides Quantified with LC-SRM on the TSQ Vantage

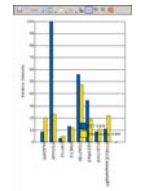


Figure 7 displays single point relative quantitation of 8 Klotho peptides, comparing the 2 hour PCT digestion and 48 hour urea control digest. Each peptide was normalized to its corresponding heavy isotope labeled internal standard. All data shown in Figure 7 was acquired with LC-SRM on the TSQ Vantage. PCT samples are shown in blue and Urea is displayed in Yellow.

Overall, peptides performed differently depending on the digestion method used. The FSISWAR peptide showed the most significant improvement with PCT. There were also several peptides that performed better with a standard urea digest. The FSISWAR peptide, however, provides the best digestion efficiency of all the peptides monitored.

FIGURE 8. Other Peptides Identified/Quantified with LC-Full Scan on the Exactive

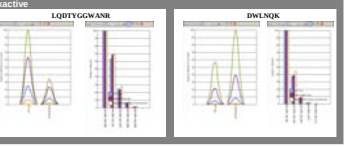


Figure 8 displays data acquired on the Exactive mass spectrometer. The PCT and Urea digestion samples were run on the Exactive using the LC described in the Methods section. The screening tool available in Pinpoint software allowed for the evaluation of Full Scan accurate mass and isotopic distribution to identify and quantify all peptides present in the samples. Results show that the LQDTYGGWANR peptide was 2.5X more abundant in the PCT sample and the DWLNQK peptide was 2X more abundant in the Urea sample. Both peptides shown in Figure 8 were normalized to a peptide internal standard eluting within 30 seconds of each peak. Besides the peptides in Figure 7, the peptides shown in Figure 8 had the most significant difference in abundace between PCT and Urea samples, matched theoretical isotope distribution patterns closely, were within 3 ppm of their theoretical average mass and eluted closely to what was predicted based on hydrophobicity of the sequence.

Conclusions

Individual peptides from a protein tryptic digest behave very differently depending on the digestion method that is used. PCT can significantly improve the efficiency and throughput of a tryptic digestion when monitoring the FSISWAR peptide of Recombinant Mouse Klotho.

References (optional)

1.Lopez-Ferrer, D., et al., *Application of pressurized solvents for ultrafast trypsin hydrolysis in proteomics: proteomics on the fly.* J Proteome Res, 2008. 7(8): p. 3276-81.
2.Lopez-Ferrer, D., et al., *Pressurized pepsin digestion in proteomics: An automatable alternative to trypsin for integrated top-down bottom-up proteomics.* Mol Cell Proteomics, 2010.