

Proteolysis (Trypsin)-PrEP: In-Solution PCT-Enhanced Trypsin Digestion for Proteomics

Introduction

In-solution digestion of proteins is a standard method used in proteomics that is suitable for quantitative analysis by multidimensional chromatography coupled with tandem mass spectrometry. Traditional methods for in-solution digestion typically rely on high concentrations of chaotropic reagents for protein denaturation and time consuming enzymatic steps to ensure good peptide coverage and accurate protein identification from complex proteomic samples [1]. Several groups have shown that pressure cycling technology (PCT) facilitates acceleration of protein digestion and that PCT acts synergistically with other protein denaturation methods, such as using organic solvents and elevated temperature to maintain proteins in their denatured state during digestion. PCT-enhanced tryptic digestion substantially reduces processing time and increases sample throughput, while it provides higher sequence coverage, resulting in more accurate protein identification by mass spectrometry (See Figure 1). Successful in-solution digestion of single proteins and complex protein mixtures were achieved in 60 seconds. This was followed by analysis using reversed phase liquid chromatography-electrospray ion trap-mass spectrometry [2]. Other laboratories report the time of digestion was reduced from hours to 9-45 minutes depending on the method used [3, 4]. In each case, the use of the PCT SPS significantly reduced sample preparation and digestion time from hours to minutes, while improving the quality of protein identification.

PCT Sample Preparation System (PCT SPS)

Pressure Cycling Technology Sample Preparation System (PCT SPS) uses rapid cycles of hydrostatic pressure between ambient and ultra high levels to control biomolecular interactions. The PCT SPS can be used to disrupt tissues, cells, and cellular structures to extract proteins and nucleic acids. [5]. In addition, the PCT SPS can also be used to accelerate enzymatic reactions such as trypsin digestion. The PCT SPS uses a small, semi-automated bench top instrument (Barocycler NEP3229 or the NEP2320) in combination with single-use sample processing containers called FT500-ND PULSE Tubes (Pressure BioSciences, Inc. South Easton, MA). The combination of PCT and the FT500-ND employs rapid pressure changes, chemistry, and other biophysical mechanisms to accelerate trypsin digestion and other enzymatic reactions.

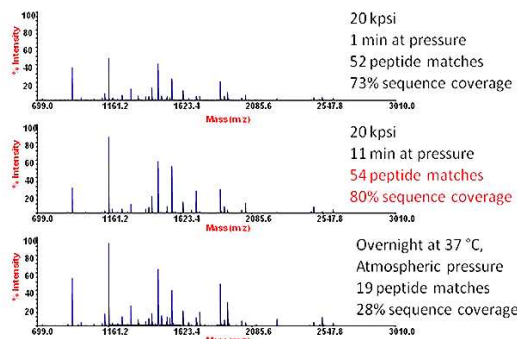


Figure 1. 100ng of BSA was digested in 200 μ L of 25 mM ammonium bicarbonate; modified porcine trypsin was added at 1:50 enzyme-to-substrate ratio. PCT was conducted at 20,000 psi and held for 60 seconds per cycle for 1 or 11 cycles. Digests were analyzed on an ABI 4700 MALDI-TOF/TOF instrument in a reflectron mode using α -cyano hydroxycinnamic acid as matrix. Control overnight incubation at atmospheric pressure shows more trypsin autolytic peaks and lower sequence coverage than the 11 minute PCT run.

Protein Denaturation, Reduction, and Alkylation

PCT-enhanced protein digestion is a thermodynamic approach for acceleration of enzymatic activity and improvement of protein substrate availability. While PCT is fully compatible with conventional protein denaturation methods that require high concentrations of chaotropic reagents, such as urea and guanidine, hydrostatic pressure cycling promotes protein denaturation. PCT allows for the decrease or, in some cases, the elimination of chemical denaturants. This approach greatly simplifies the digestion procedure and minimizes protein loss. Alternating hydrostatic pressure, a combination of elevated temperature and organic solvents (e.g. methanol/acetonitrile [6] or trifluoroethanol [7]) act synergistically to denature proteins. However, it should be noted that while the presence of organic solvents such as methanol or acetonitrile will accelerate trypsin digestion, concentrations of these solvents above 20% at high hydrostatic pressure may lead to partial trypsin denaturation and a decrease in proteolytic activity. Reduction and alkylation of disulfide bonds is optional and may be omitted. However, peptide coverage of non-reduced and non-alkylated proteins may be significantly lower than of those proteins which have been reduced and alkylated. If confirmation of the presence of these bonds is required, reduction and alkylation of proteins prior to digestion may be done using a number of standard protocols that may already be in practice in your laboratory. For example, PCT-enhanced proteolysis has been demonstrated with reduction/alkylation protocols using dithiothreitol/iodoacetamide [1], tributylphosphine/acrylamide [8] and TCEP/vinylpyridine methods [9]. Proteins prepared by the methods described in this section are now ready for PCT-enhanced trypsin digestion.

PCT-enhanced Trypsin Digestion

PCT-enhanced trypsin digestion may be performed in aqueous 25-50 mM ammonium bicarbonate (pH=8.0) or in 20% (v/v) aqueous methanol or acetonitrile containing 25-50 mM ammonium bicarbonate. Add the trypsin solution to achieve a final protease-to-protein ratio between 1:20 and 1:50 (w/w), and transfer the solution to a FT500-ND PULSE Tube (total volume between 0.2 and 1.4 mL). Place the PULSE Tube into a Barocycler equipped with a circulating water bath pre-heated to 37°C. The standard pressure cycling program for trypsin digestion is as follows: 20 pressure cycles, where each cycle consists of 50 seconds at 20,000 psi and 10 seconds at ambient pressure. (NOTE: the standard pressure cycling program just outlined can be optimized by each user by changing the number of total cycles, the high pressure limit, and the time at pressure – please refer to the information in the “Discussion” section below). Following PCT, the digestion reaction can be stopped by the addition of either 100 mM acetic acid, 1-5% formic acid, or 0.1% TFA (final concentration) depending on the chosen downstream method of analysis. Alternatively, the samples can be rapidly frozen to stop trypsin digestion. The samples are now ready for downstream analysis.

Discussion

It has been reported that thermodynamic treatments, such as High Intensity Focused Ultrasound (HIFU) [10], microwave radiation [11] and high pressure [12, 13], can facilitate and accelerate digestion by trypsin and other enzymes. Several laboratories have shown that pressure cycling technology (PCT) can be used to accelerate tryptic digestion while providing good peptide coverage, resulting in more accurate protein identification [2, 3, and 4]. In the approach described herein, pressure cycling not only accelerates the trypsin-catalyzed hydrolytic reactions [12], but it acts synergistically with organic solvents to dynamically alter protein conformation and to maintain protein substrates in their denatured state, thus obviating the need for high concentrations of chaotropic reagents and long incubation times [13]. Subsequently, incubation times have been reduced from hours to minutes allowing for increased throughput and analysis of more samples (See Figure 1). However, due to the diversity of proteolytic digestion approaches and to the complexity of proteomic samples, the method described here should be considered as a starting point for optimization. For example, variations in pressure from 10,000 psi to 25,000 psi, total time at pressure, and number of pressure cycles may be used to further optimize trypsin digestion for a chosen set of denaturants, reduction and alkylation reagents, enzyme preparations, and digestion buffers compositions. By varying these parameters, it may be possible to reduce the digestion time of difficult-to-digest samples to five minutes or less, with no degradation in quality. Several groups have reported digestion times ranging from 1–45 minutes [3, 4] for model proteins and complex samples. Furthermore, one group reported that since some trypsin digestions can be conducted at room temperature, undesired protein modifications can be minimized [2]. The same group concluded that “PCT simplified sample preparation compared with other newer rapid digestion methods, such as MAPED (microwave-assisted digestion) and HIFU (high intensity focused ultrasound) technologies.” Researchers from another group [4] reported that “The results from these experiments show the utility of PCT for rapid enzymatic digestion of samples. This technique is ideal for high-throughput situations and for the rapid analysis of biological agents where timely identification is paramount.” Both groups highlighted other advantages of the PCT SPS such as automated sample preparation. It was also noted that the PCT SPS produced no aerosolization (a common undesirable effect with other techniques). In summary, data from our laboratory and independent customer publications show that the PCT SPS is an effective method of accelerating trypsin activity with high reproducibility but without compromising digestion yields.

References

1. Kinter M., Sherman N. Experiments In Protein Sequencing and Identification Using Tandem Mass Spectrometry, Series Editor(s): Dominic M. Desiderio, Nico M. M. Nibbering, John Wiley and Sons, Inc. ISBN: 9780471322498, p. 147-165, 2005
2. López-Ferrer D., *et al.*, J Proteome Res. 2008 Jul 8. [Epub ahead of print]
3. Pevsner P., *et al.*, Poster. LC-MS Course and Symposium. Robinson College Cambridge, UK. December 9–13, 2007
4. Croley, T.R., Poster. ASMS, May 20, 2008
5. Schumacher R.T., *et al.*, (2002). Am. Laboratory 34, 38-43.
6. Strader M.B., *et al.*, Anal Chem. 2006 Jan 1;78(1):125-34.
7. Ru QC., *et al.*, J Chromatogry 2006 Apr 14;1111(2):175-91.
8. Andrews, P.C., Dixon, JE. Anal. Biochem. 161 (1987) 524–528.
9. Bai F., *et al.*, Proteomics. 2005 May; 5(8):2043-7.
10. López-Ferrer D., Capelo J.L., Vázquez J., Res. 2005 Sep-Oct;4(5):1569-74.
11. Pramanic B.N., *et al.*, Protein Science. 2002, 11(11):2676-2687.
12. Mozhaev V., *et al.*, Biotechnology and Bioengineering, 1996, 52:320-331
13. Chicon R., *et al.*, Journal of Dairy Research, 2006; 73:121-128