Guidelines for Protocol Development: Rapid Trypsin Digestion of Proteins in Solution Using Pressure Cycling Technology

Introduction

Pressure-enhanced proteolytic digestion exploits the ability of high hydrostatic pressure to promote protein denaturation and the access of proteolytic enzymes to their target sites. Pressure denaturation is fundamentally different from thermal denaturation as it occurs by virtue of hydration of hydrophobic residues and by water saturation of protein substrate cavities normally inaccessible to solvent. Pressure denaturation is more efficient for hydrophobic proteins, while some hydrophilic soluble proteins are reported to retain relatively compact conformation, even when saturated by water molecules [1-6].

Since pressure alone may not be sufficient to denature some stable proteins, particularly proteins cross-linked with disulfide bridges, common reducing agents can be used to help promote pressure-induced denaturation. Of course, when the goal of the experiment is to verify the presence of disulfide bridges, reducing reagents should be omitted, while low concentration of chaotropic denaturants may be beneficial. The destabilizing effects of most common chaotropic agents and organic solvents are greatly enhanced under hydrostatic pressure. While this phenomenon presents opportunities to decrease the use of these reagents to simplify post-digestion sample clean-up and compatibility with downstream analytical steps, care must be taken to avoid detrimental effects of chaotropic reagents on the trypsin or other protease used for digestion under pressure.

Pressure-enhanced digestion of highly soluble and easy-to-digest proteins (e.g., BSA) may not be substantially different from standard digestion. The **most significant enhancements** are generally observed with **hydrophobic proteins** (e.g., those derived from membranes or organelles) or other difficult-to-digest targets (e.g., ubiquitin or native IgG). Pressure-enhanced trypsin digestion of these types of samples typically leads to identification of a significantly higher number of peptides and proteins with substantially higher sequence coverage.

Pressure Cycling Technology (PCT)

In the Pressure Cycling Technology Sample Preparation System (PCT SPS) hydrostatic pressure is rapidly cycled between ambient and ultra high levels (up to 45,000 psi) to control biomolecular interactions. High hydrostatic pressure is used to accelerate enzymatic digestion of proteins, as well as to disrupt animal and plant tissues, cells, cellular structures and microbes to extract proteins, nucleic acids, and lipids. The system is comprised of a small, semi-automated bench-top instrument (Barocycler NEP2320, NEP3229 or HUB440) used in combination with single-use sample processing containers (MicroTubes or PULSE Tubes).

Methods

1. The recommended starting **instrument program** for pressure-enhanced trypsin digestion is 20,000 psi (50 seconds at high pressure, 10 seconds at ambient pressure per cycle) repeated for at least 60 cycles. These conditions can then be optimized for each individual application. If the initial digestion results are found to be acceptable, the number of cycles could be reduced to save time. In some cases, 30 cycles or fewer may be sufficient. In other cases, 120 or even 180 cycles may be necessary to obtain adequate digestion of difficult-to-digest targets. The use of more rapid pressure cycling has also been shown to be beneficial in some cases. If the above parameters do not yield satisfactory results, pressure cycling at 20,000psi using 20 seconds at high pressure, 10 seconds at ambient pressure per cycle (for 90-120 cycles) may be of benefit. Note that the more rapid cycling parameters may lead to more rapid inactivation of trypsin. Therefore, these conditions should be used only if the standard parameters are found to be unsatisfactory.

2. The recommended **pressure** for trypsin digestion is 20,000 psi. Higher pressures may be used, but may lead to more rapid trypsin inactivation.

3. The recommended reaction **volume** is 50 µL per MicroTube. Under-loading MicroTubes (e.g., using only 30µL of liquid with the long 50 µL caps), may result in tube deformation and difficulty retrieving the sample, but not loss of sample. In case of severe tube deformation, a thin gel loading pipet tip can be used to retrieve the sample from the bottom of the MicroTube.

4. The recommended **temperature** is 50˚C. High pressure is thought to protect proteins from thermal denaturation, therefore, optimal trypsin activity at high pressure can be maintained at much higher temperatures. Note that for digests containing urea, the temperature should be kept at or below 37℃ to avoid protein carbamylation. Lower temps may be used, but digestion time may need to be increased for optimal results. Temperatures significantly above 50˚C should be avoided as these could lead to trypsin inactivation.

5. The recommended **buffer** for pressure-enhanced trypsin digestion is 25-50mM ammonium bicarbonate (pH between 8 and 8.5). Note that under pressure the pH may shift down by as much as 0.5 pH units.

6. The addition of certain **denaturants** is compatible with pressure cycling and may lead to improved sequence coverage. However, the commonly employed concentrations of these reagents may be detrimental to trypsin activity at high pressure since their chaotropic action is greatly enhanced by pressure. It may therefore be necessary to reduce the concentration of these reagents, or to omit them entirely, when performing pressure-enhanced trypsin digestion. The following is a partial list of commonly used additives and their compatibility with high pressure digestion:

    a. The addition of 5-10% **n-propanol** is recommended for pressure-enhanced trypsin digestion. However, higher concentrations result in loss of trypsin activity and should be avoided.
    b. The addition of up to 10% **acetonitrile** is compatible with pressure-enhanced trypsin digestion at 20,000 psi. However, higher concentrations may interfere with trypsin activity.
    c. **Mass-spectrometry-friendly detergents** (Rapigest, ProteaseMax, Progenta surfactants) can be used under pressure. The optimal concentrations of these reagents may need to be determined empirically, but are likely to be similar to, or less than, those recommended by their respective vendors for digestion at ambient pressure.
    d. The use of **urea** in the trypsin digest should be avoided. If urea is already present in the sample, it should be diluted to 0.5M or less and the digestion temperature should be reduced to 35-37˚C to minimize carbamylation.
    e. The use of high concentrations of **methanol** in the trypsin digest should be avoided. If methanol is present in the sample, it should be kept at or below 10%.
    f. The use of **guanidine hydrochloride** in the trypsin digest should be avoided. If guanidine hydrochloride must be used, its final concentration should not exceed 100 mM to avoid trypsin inactivation.
    g. **Trifluoroethanol** (TFE) at concentrations up to 10% is compatible with pressure-enhanced trypsin digestion. However, concentrations above 15% result in loss of trypsin activity and should be avoided.
    h. Carry-over of residual **ProteoSolve-SB Reagent A** should be minimized to below 4% final concentration.
    i. **CHAPS** in concentrations as low as 0.2% has been shown to interfere with pressure-enhanced trypsin digestion. Other detergents have not been tested, but are expected to also interfere with trypsin under pressure.

General References

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PCT-Specific References

Pressure-Enhanced Enzymology
http://www.pressurebiosciences.com/pub/pressure-enhanced-enzymatic-proteolysis-publications.html