"P3" Sample Prep in the ProTrap XG: Precipitate, Purify, & Pepsin Digestion

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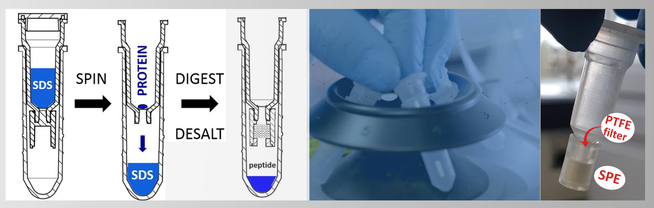


OBJECTIVES

Optimize protein digestion with pepsin in presence of formic acid.

Assess proteome resolubization efficiency with formic acid.

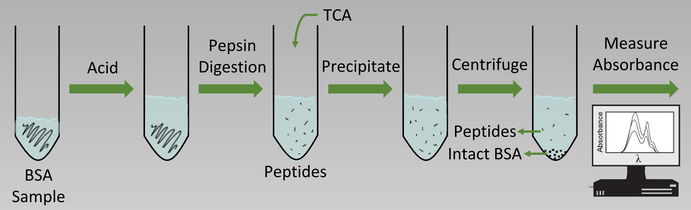
Establish an efficient workflow for bottom-up analysis of proteins that are precipitated in the ProTrap XG.

INTRODUCTION

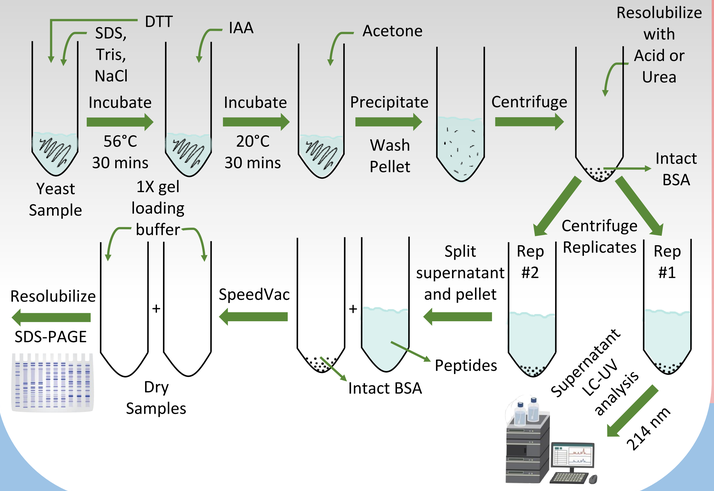
Though trypsin is favored for bottom-up proteomics workflows, alternative enzymes including pepsin have been employed to improve sequence coverage, or in instances where basic digestion conditions are not possible (eg HDX). Pepsin digestion is conventionally performed in dilute HCl to mimic the natural low pH environment of the stomach. In prior work, we showed that formic acid is ideally suited to resolubilize precipitated proteins, being recommended for top-down proteomic workflows. Here, we establish a complete proteomics workflow within a single spin-cartridge device known as the ProTrap XG. Proteins can be extracted from cells, purified by precipitation, resolubilized in concentrated formic acid, digested with pepsin, and subject to further fractionation, all within the device, ahead of MS.

METHODS

Pepsin HCl/Formic Acid Enzyme Activity Assays



Protein Recovery After Precipitation and Resolubilization



RESULTS

Pepsin HCl/Formic Acid Activity Assays

Optimal pepsin activity was observed in 5% formic acid (Figure 1) and in pH 1.8 HCl (Figure 2). Figure 2 also shows that pH 1.9 maximizes pepsin activity in formic acid

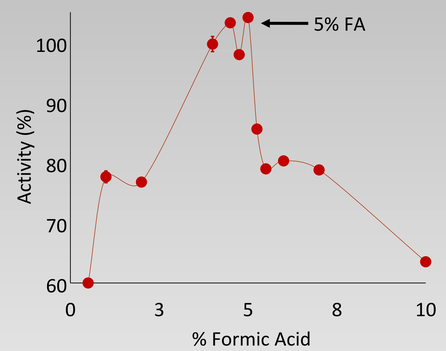


Figure 1 Pepsin formic acid activity assay curve as a function of concentration.

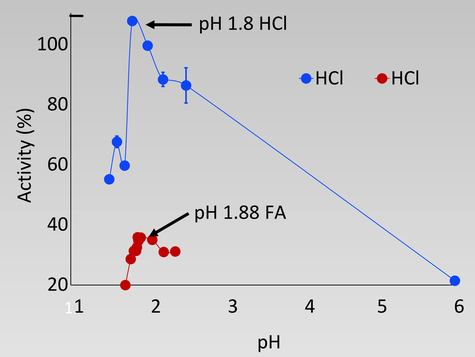


Figure 2 Pepsin HCl and formic acid activity assay curves as a function of pH.

SDS PAGE Visualization of Pepsin Digestion

Formic acid is a better solvent than HCl for digestion (Figure 3C). As shown in Figure 3, allowing pepsin to digest BSA for > 1 hour in 5% formic acid results in the least intact proteins and more peptide fragments.

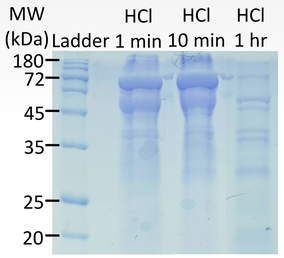


Figure 3A SDS PAGE (15% T) gels comparing pepsin digestion times in pH 1.8 HCl.

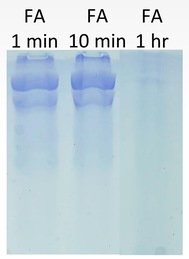


Figure 3B SDS PAGE comparing pepsin digestion times in 5% formic acid.

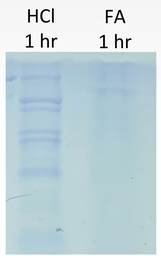


Figure 3C SDS PAGE comparing pepsin digestion in HCl vs formic acid.

Qualitative and Quantitative Analysis of Intact Protein Recovery

Formic acid (5%) is a better solvent than HCl (pH 1.8) to resolubilize proteins (Figures 4 and 5).

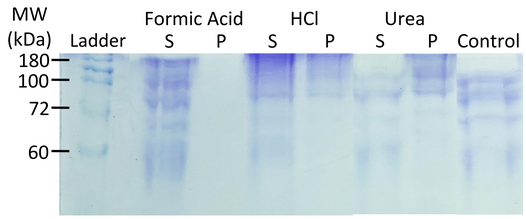


Figure 4 SDS PAGE comparing solvents formic acid, HCl, and urea at resolubilizing proteins. "S" represents dissolved yeast, and "P" represents the yeast pellet.

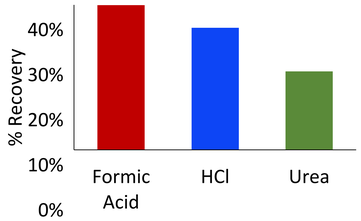


Figure 5 LC-UV peak area analysis comparing solvents formic acid, HCl, and urea at resolubilizing yeast.

CONCLUSIONS

- Pepsin's optimal conditions: pH 1.8 HCl and 5%/pH 1.88 formic acid.
  - As the pH/concentration of HCl and formic acid deviates from its optimal condition, pepsin activity drops significantly.
- Results from activity assays and SDS PAGE gels appear to contradict each other.
  - The assays are good within themselves but are not accurate at showing whether pepsin works best in HCl or formic acid.
  - Formic acid presence "confuses" the solubility of peptides in TCA.
- Digestion with pepsin under optimal conditions requires > 1 hr to cleave intact proteins sufficiently for optimal bottom-up MS.
- Formic acid is a better solvent than HCl.
  - Formic acid provides superior pepsin activity.
  - Formic acid resolubilizes a precipitated pellet more efficiently.

FUTURE WORK

- Mass spectrometry data analysis should be completed to examine proteins and quantify the peptides found in solution after pepsin digestion in HCl and formic acid.
- Different protein samples should be analyzed for protein resolubilization by HCl and formic acid.
  - Hydrophobic yeast membrane protein samples would be challenging to redissolve.
  - Lactobacillus protein samples.

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