Oxidation Effects on Chymotrypsin Digested HRPF Samples and Observation of Highly Basic Regions

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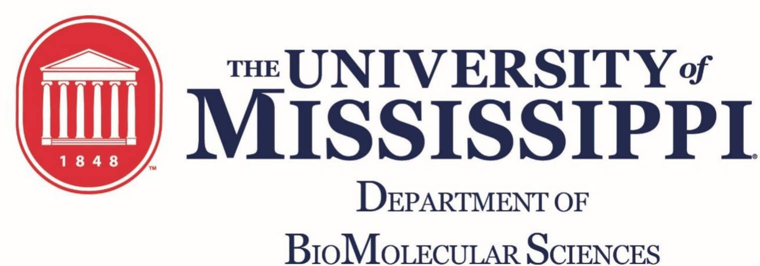


Overview

- Proteins that bind heparin/heparan sulfate (Hp/HS) usually posses highly basic regions (HBRs)
- Hydroxyl radical protein footprinting (HRPF) can be used to identify protein-carbohydrate binding sites
- Trypsin is commonly used to generate peptides for LC-MS/MS analysis of HRPF samples
- Trypsin digestion of HBRs results in peptides too small for reliable identification and quantification
- Chymotrypsin can digest HBR containing proteins and generate peptides large enough for reliable identification, but the effects of oxidized substrate on chymotrypsin cleavage patterns have not been studied in detail.
- Oxidation affects chymotrypsin digestion patterns more than trypsin in lysozyme and RPTP-Sigma, probably due to oxidation near the digestion site
- Care must be taken when analyzing HRPF samples digested with chymotrypsin

Introduction

The interaction of Hp/HS, a family of highly sulfated glycosaminoglycans, with the leukocyte common antigen-related (LAR) subfamily of receptor protein tyrosine phosphatases (RPTPs) has been shown to control neurite growth and regeneration in animal models. The interaction is believed to occur at HBRs[1], 42-KKGKKVNSQR-51 (RPTP-LAR) and 39-KKGKKVNSQR-48 (RPTP-Sigma), within the first Ig-like domain, making observation of the site after trypsin digestion at K and R using standard hydroxyl radical protein footprinting (HRPF) methods impossible. Chymotrypsin digestion preserves the proposed site within peptides large enough for correct identification and increases the overall sequence coverage. Here we test the effect of oxidation on the efficiency of chymotrypsin digestion for the preservation of highly basic regions.

Methods

RPTP-Sigma and lysozyme were mixed to a final concentration of 4uM in standard FPOP buffer, with/without 4uM porcine intestinal mucosa HP (molecular weight 15,000 +/- 2,000). HRPF via fast photochemical oxidation of proteins (FPOP) was carried out in quadruplicate in sodium phosphate buffer, quenched into a final buffer of 100mM Tris, 10mM $CaCl_2$, pH 8.0. $CaCl_2$ was replaced to 4.4mM after precipitation due to phosphate presence in the FPOP buffer. Samples were digested with trypsin or chymotrypsin for 12 hours and analyzed by LC-MS/MS (Orbitrap Fusion) using a targeted approach. The PEP2D score from Byonic analysis, anything scoring at or below 0.01 being accepted, was used to determine the number of spectrum observations for each peptide.

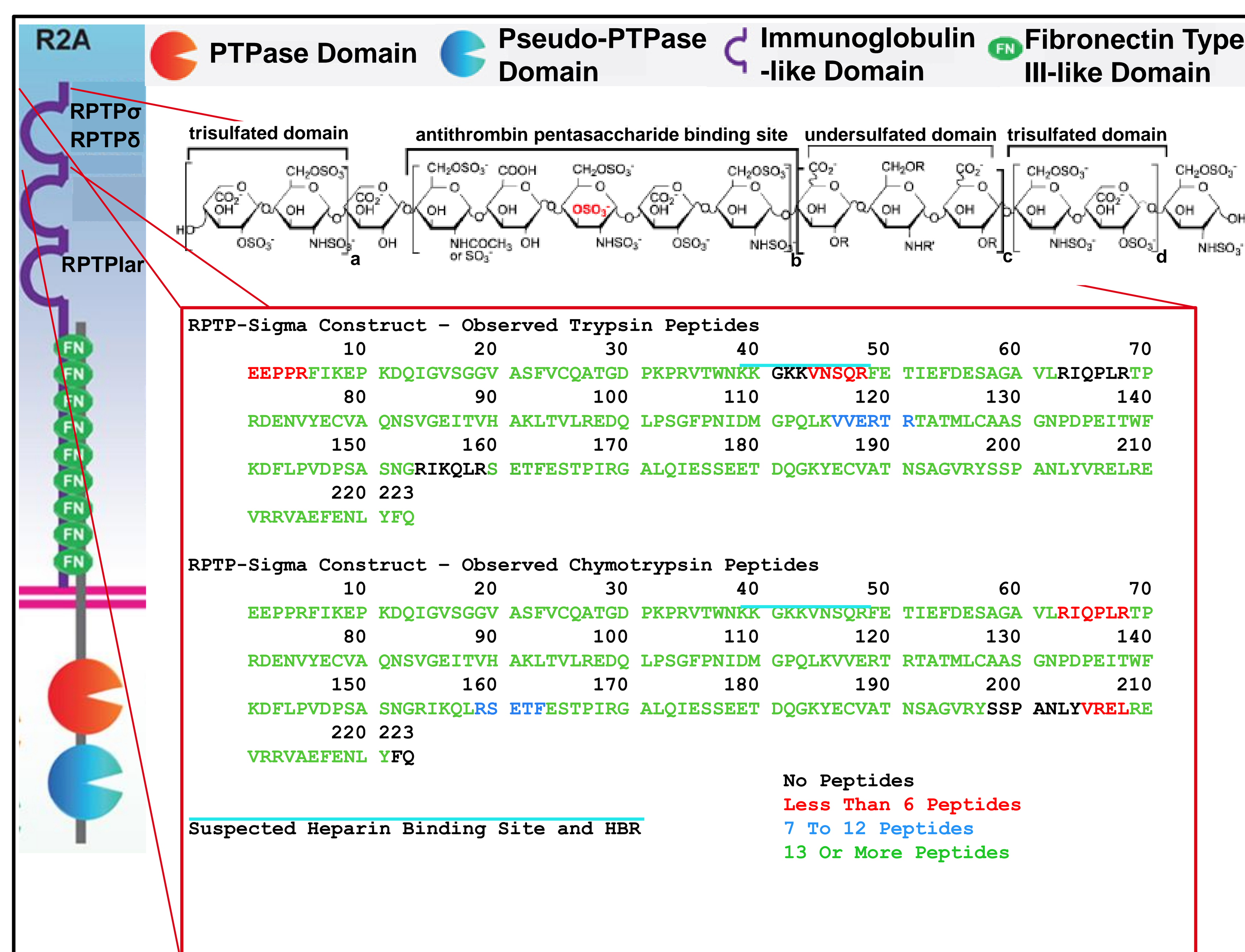
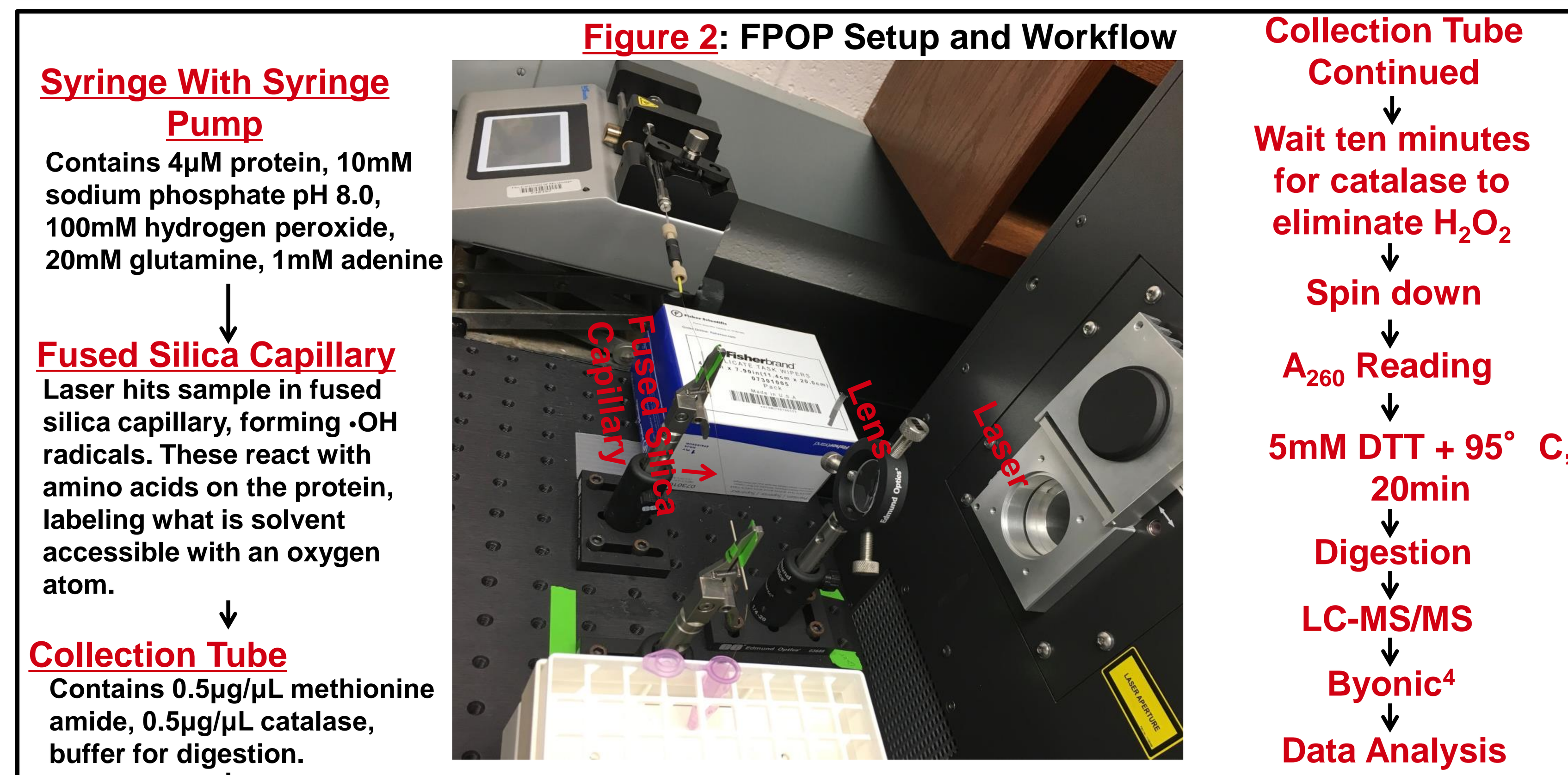


Figure 1: Left and Top. Type II RPTP cartoon model.[2] Middle. Structure of Heparin Dissacharide repeat.[3] Red Square. Sequence of the immunoglobulin-like domains from RPTP-σ made and used for Hp binding studies. Amino acids colored to show how often they appear on average when digested by different proteases.

Figure 2: FPOP Setup and Workflow



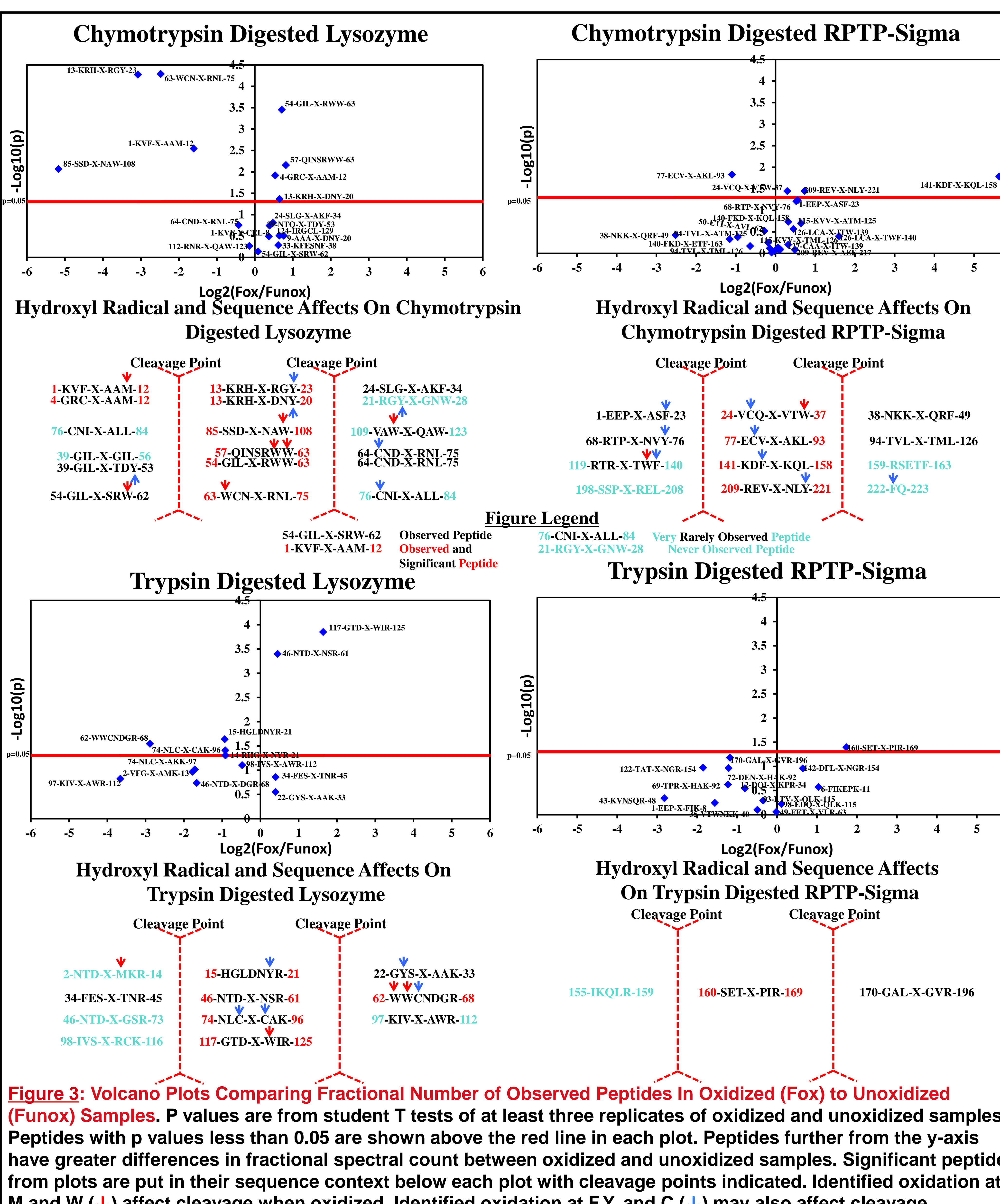


Figure 3: Volcano Plots Comparing Fractional Number of Observed Peptides In Oxidized (Fox) to Unoxidized (Funox) Samples. P values are from student T tests of at least three replicates of oxidized and unoxidized samples. Peptides with p values less than 0.05 are shown above the red line in each plot. Peptides further from the y-axis have greater differences in fractional spectral count between oxidized and unoxidized samples. Significant peptide from plots are put in their sequence context below each plot with cleavage points indicated. Identified oxidation at M and W (↓) affect cleavage when oxidized. Identified oxidation at F,Y, and C (↓) may also affect cleavage.

$F_{ox} = ((Pep_{ox}/APep_{ox})_{r1} + (Pep_{ox}/APep_{ox})_{r2} + (Pep_{ox}/APep_{ox})_{r3})/n$

$F_{unox} = ((Pep_{unox}/APep_{unox})_{r1} + (Pep_{unox}/APep_{unox})_{r2} + (Pep_{unox}/APep_{unox})_{r3})/n$

Pep_{ox} = Peptide spectral count from oxidized replicate
$APep_{ox}$ = Sum all peptide spectral counts from oxidized replicate
Pep_{unox} = Peptide spectral count from unoxidized replicate
$APep_{unox}$ = Sum all peptide spectral counts from unoxidized replicate
n = Number of Replicates
r1 = replicate 1, etc.

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        10         20         30         40
KVFGRCELAA AMKRHGLDNY RGYSLGNWVC AAKFESNFNT
        50         60         70         80
QATNRNTDGS TDYGILQINS RWWCNDGRTP GSRNLCNIPC
        90        100        110        120
SALLSSDITA SVNCAKKIVS DGNGMNAWVA WRNRCKGTDV
       129
QAWIRGCRL
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Figure 4: Sequence of chicken egg white lysozyme used as a control for the comparison of chymotrypsin and trypsin digestion.[5] Same color coding of observed peptides as in Figure 1.

Conclusions

- Chymotrypsin can cleave near sites of oxidation
- Chymotrypsin does provide peptides that cover HBRs
- Oxidation at M and W affects chymotrypsin cleavage
- Oxidation on F, Y, and C may also affect chymotrypsin cleavage
- Oxidation may also affect trypsin cleavage to a much lesser extent
- No obvious sequence connection to trypsin cleavage effect – need more data

References

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