PRESSURIZED ONLINE PEPSIN DIGESTION OF PROTEINS FOR HYDROGEN/DEUTERIUM EXCHANGE MASS SPECTROMETRY

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INTRODUCTION

- Online pepsin digestion has been widely utilized in hydrogen/deuterium exchange mass spectrometry (HDX MS) because of its high digestion reproducibility; minimized introduction of pepsin autolysis fragments into LC/MS system, etc. However, typical online pepsin digestion may not generate sufficient digestion for some proteins. In this study, we report an improvement in protein digestion using a high pressure approach.

- High pressure promotes protein denaturation, which mechanically stretches proteins to expose more cleavage sites. Here, a high-pressure sustainable BEH column with immobilized pepsin [1], was applied in the digestion of monoclonal antibodies. The digestion efficiency of this column under high pressure (~12,000 psi) and under normal pressure (~ 1000 psi) was compared under different digestion conditions. More overlapping peptic peptides were observed for IgG2 under enhanced pressure, which substantially increases the protein sequence coverage and redundancy score. Furthermore, the peptides generated under high pressure are shorter in length, which also improves the spatial resolution. In addition, the destabilizing effects of the chaotropic agents are enhanced under higher pressure. Less chaotropic reagents could simplify the post-digestion and are more compatible with the downstream analytical steps.

METHODS

Samples: IgG2 (denosumab, Amgen) was prepared by dilution of protein stock 15-fold (v/v) with equilibrium buffer. The labeling reaction were quenched with an equal volume of pre-chilled quench buffer. The totally deuterated angiotensin II and bradykinin (Sigma) peptides were prepared as described before [2]. This fully deuterated mixture was used to determine the loss of deuterium in the system.

System: Quenched samples were subjected to online pressurized pepsin digestion. High pressure was generated by a prototype Back Pressure Regulator (BPR) as shown in Figure 1. Waters M-class UPLC system with HDX Manager was used for rapid peptide separation at 0 °C. Peptides were directed into Waters Synapt G2-S HDMS system. Peptic peptides were first identified using Waters ProteinLynx Global Server (PLGS 3.0.2). Peptide mapping with peptide numbers and redundancy score were generated by in Waters DynamX 3.0. The workflow is shown in Figure 2.

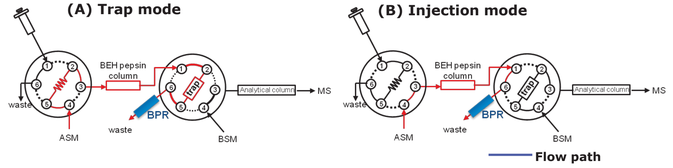
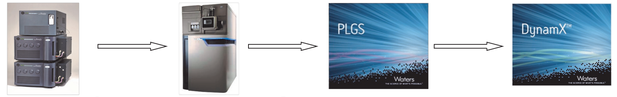


Figure 1. *Plumbing scheme for the flow path in Trap (A) and Injection (B) modes. The red line indicates the flow path. The back pressure regulator (BPR) are colored in blue. ASM, auxiliary solvent manager, delivers mobile phase [0.1% FA in H_2O pH 2.5] for pepsin digestion and BSM, binary solvent manager, drives the reversed phase gradient [0.1% FA in H_2O and 0.1% FA in ACN] for peptide trapping and separation.*



ACQUITY UPLC® M-Class HDX System | **Synapt G2-Si HDMS^E** | **PLGS 3.0.2** | **DynamX 3.0**

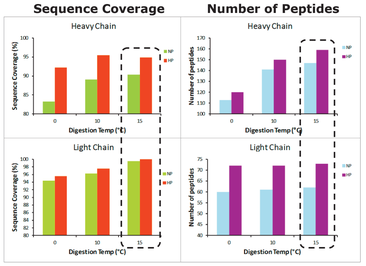
Figure 2. ***Experimental workflow***

RESULTS AND DISCUSSION

Optimizations and comparisons of digestion efficiency of IgG2 under normal and enhanced pressures

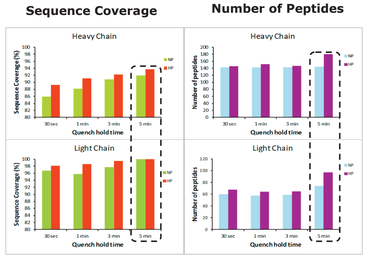
(A) Digestion temperature

- *Experimental conditions: quench buffer contained 4 M GdnHCl and 0.5 M TCEP, quench hold time was 5min, and loading flow rate was 200 µL/min for 4min.*
- *Results: enhanced digestion efficiency was obtain at higher temperature and 15 °C was chosen for the following experiments.*



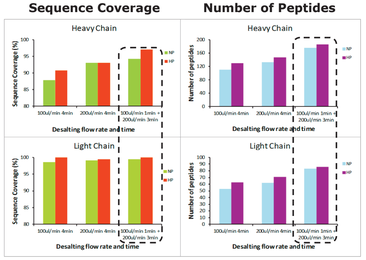
(B) Quench hold time

- *The reaction time of denaturation and reduction after adding quenching buffer is called quench hold time.*
- *Experimental conditions: quench buffer contained 4 M GdnHCl and 0.5 M TCEP, quench hold was performed at 0°C, and loading flow rate was 200 µL/min for 4min.*
- *Results: The sample was held at 0 °C for 5 min to obain best digestion efficiency for IgG2 with normal backexchange (see Figure 6 (A)).*



(C) Flow rate and digestion/desalting time

- *Higher flow rate and longer desalting time give cleaner MS background, especially for the quench buffer containing high conc. of salts.*
- *Results: Compromising with backexchange (see Figure 6. (C), in the following experiments pepsin digestion was performed at 100 µL/min for 1min and 200 µL/min for the remaining 3 min.*



(D) Chaotropic reagents

- *Different concentration of Guanidine HCl and TCEP in quench buffer were tested.*
- *Results: Comparable, or slightly better, digestion efficiency was obtained under HP using less TCEP (0.4 M) and GdnHCl (3 M), comparing with the one under NP using more TCEP (0.5 M) and GdnHCl (4 M).*

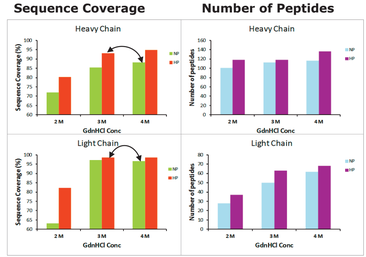


Figure 3. *Sequence % coverage (left column) and # of peptides (right column) of IgG2 (heavy chain, upper and light chain, bottom) were determined and compared at various digestion conditions under normal pressure (NP) and higher pressure (HP). The conditions that were chosen for the following experiments are highlighted in dashed box.*

The effects of pressurized digestion on back exchange

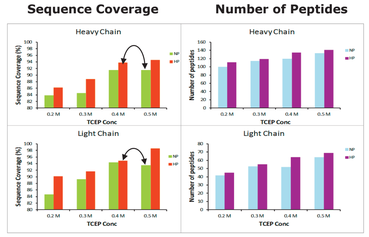


Figure 4. *Sequence map of heavy chain of IgG2. The peptides identified under NP is shown as cyan bars. Unique peptides generated by HP digestion are shown in yellow.*

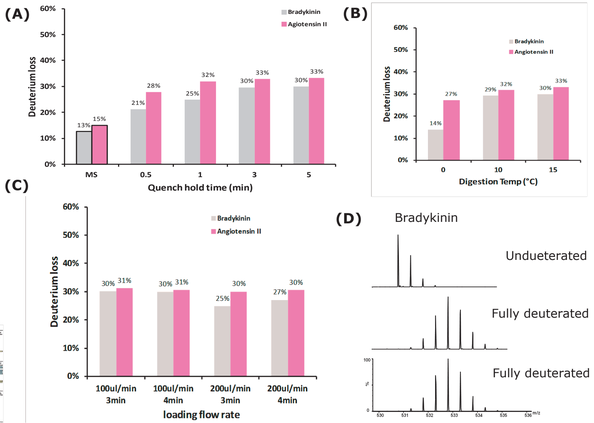


Figure 5. *Comparison of deuterium loss from fully deuterated Bradykinin and Agiotensin II at various quench hold times (A); digestion temperature (B); and desalting/loading flow rate and time (C). The deuterium loss caused by mass spectrometer is highlighted in black border in (A). Similar isotope profile were found in both peptides under NP and HP. HP caused a slightly higher deuterium loss. A representative mass spectra are shown in (D).*

CONCLUSIONS

- **An enhanced pressure (up to ~ 15,000 psi) sustainable BEH column with immobilized pepsin has been successfully applied in Waters HDX MS system.**
- **An systematically study of protein digestion efficiency and back exchange under enhanced pressure shows an improvements in mAb IgG2 digestion efficiency. IgG2 is believed to be resistant to digestion since four disulfide bonds locates in the hinge region.**
- **Our results indicated:**
  - **Overall more overlapping peptides with shorter length were observed under HP digestion, which increased protein sequence coverage, and spatial resolution compared to NP under the same digestion conditions.**
  - **Relatively high temperature (15 °C) and longer quench hold time (5 min) increased the sequence % coverage of IgG2, especially for heavy chain, without causing significant deuterium loss.**
  - **Higher flow rate and longer desalting time minimized the salt contamination without causing more deuterium loss.**
  - **Less chaotropic reagents was used under HP digestion and obtained a similar digestion efficiency.**

References

1.Ahn, J. *et al.*, *Anal. Chem.*, **2012**, 84, 7256-7262.
2.Wu, Y. *et al. Anal. Chem.*, **2006**, 78, 1719-1723.

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