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Pressurized Online Pepsin Digestion of mAb IgG2 for Hydrogen Deuterium Exchange Mass Spectrometry

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APPLICATION BENEFITS

An enhanced-pressure (up to ~ 15,000 psi), sustainable BEH column with immobilized pepsin has been successfully applied to the Waters® ACQUITY UPLC® M-Class System with HDX Technology.

Online peptic digestion of the monoclonal antibody (mAb) IgG2 led to increased digestion efficiency using high pressure (HP) when compared to ambient or normal pressure (NP).

High-pressure digestion yielded an increased number of overlapping peptides with shorter lengths, which resulted in greater protein sequence coverage and higher spatial resolution compared to normal-pressure digestion.

WATERS SOLUTIONS

ACQUITY UPLC M-Class System with HDX Technology

SYNAPT® G2-S*i* HDMS®

ProteinLynx Global SERVER™ Software

DynamX™

KEYWORDS

Higher order structure, hydrogen deuterium exchange mass spectrometry, high pressure digestion, monoclonal antibody (mAb), back pressure regulator, online digestion, pepsin column

INTRODUCTION

Pepsin digestion is an integral part of hydrogen deuterium exchange mass spectrometry (HDX-MS) that is used to obtain localized, conformational information of a whole protein. Online digestion has been widely accepted due to its high digestion reproducibility and minimized introduction of pepsin autolysis fragments into the LC-MS system. However, the typical online pepsin digestion may not generate sufficient digestion for some proteins. In this study, we report an improvement in protein digestion efficiency using a high-pressure, sustainable pepsin column – the Enzymate™ Digestion Column.

High pressure promotes protein denaturation by mechanically perturbing a protein to expose more cleavage sites. Pressurized digestion of highly-soluble and easy-to-digest proteins – such as BSA and phosphorylase B – may not yield more peptides when compared to a conventional digestion method.[1] Pressure-assisted enzymatic digestion facilitates breakdown of proteins that are resistant to proteases, particularly those with extensive disulfide bridges or abundant hydrophobic sequences. Monoclonal antibodies – especially IgG2 – are resistant to digestion under normal conditions and often require harsh denaturing conditions: 6 M guanidium hydrochloride (GdnHCl) at 100 °C and reduction using highly-concentrated dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP). However, long incubations at high temperature are not possible in HDX; HDX requires short incubation times and cold conditions to minimize back exchange. In addition, high salt concentration causes a big challenge for online pepsin digestion and interferes with MS analysis.

In this study, a commercially-available, UPLC-capable BEH column with immobilized pepsin was applied in the digestion of monoclonal antibodies IgG1 and IgG2.[2] The digestion efficiency was determined and compared under various digestion conditions at both high pressure (~15,000 psi) and typical operating pressure (~ 1,000 psi). High-pressure digestion yielded more overlapping peptic peptides with both IgG1 and IgG2. Overlapping sequences substantially increase the protein coverage and peptide redundancy score. Furthermore, high-pressure digestion resulted in shorter peptides, which improve spatial resolution. Lastly, high-pressure digestion enables more effective destabilizing effects of the chaotropic agents and permits their use in lesser concentrations that are more compatible with the downstream analytical steps.

EXPERIMENTAL

SAMPLE PREPARATION

IgG2 (denosumab, Amgen) was prepared by dilution of protein stock (~12 pmol/µL) 15-fold (v/v) with equilibrium buffer (50 mM phosphate, 100 mM NaCl, pH 6.8). The labeling reaction was quenched with an equal volume of pre-chilled 200 mM phosphate buffer with 0.5 M TCEP, 4 M GdnHCl at pH 2.3. The totally deuterated angiotensin II (Sigma) and bradykinin (Sigma) peptides were fully labeled in 99.9% deuterium oxide containing 0.5% deuterium chloride at 37 °C overnight. This fully deuterated mixture was used to determine the loss of deuterium in the system.

HDX EXPERIMENTAL METHOD

mAb IgG2 was used as the standard protein for online digestion with and without pressurization. Online pepsin digestion was performed using an ACQUITY UPLC M-Class System with HDX Technology. A series of experiments were designed to compare the digestion efficiency and deuterium back exchange of IgG2 under different digestion conditions in high/normal pressures. Essentially, the protein samples were passed through the Enzymate column (15 °C) at 200 µL/min for four minutes in 0.1% formic acid at pH 2.5 (default setting). A Back Pressure Regulator (BPR) was placed in the waste line (Figure 1) to generate enhanced pressure (up to 15 kpsi). The digests were trapped and desalted online using an ACQUITY UPLC BEH C_{18} VanGuard™ Pre-Column at 0 °C. The flow was diverted by switching valves. Trapped peptides were eluted onto an ACQUITY UPLC BEH C_{18} Column held at 0 °C. Peptides were separated under a seven-minute, linear acetonitrile gradient (2–40%) containing 0.1% formic acid at 40 µL/min. The eluent was directed into a SYNAPT mass spectrometer with electrospray ionization and lock-mass correction using the peptide Glu-Fibrinogen. Mass spectra were acquired in MS^E mode over the *m/z* range of 50–2000. Peptides were identified using ProteinLynx Global Server software (PLGS 3.0.2). All data was processed by DynamX 3.0.

ACQUITY UPLC M-Class settings

Digestion:	Enzymate Pepsin Column, 5 µm 2.1 mm x 30 mm (P/N 186007233)
Trap:	ACQUITY UPLC BEH C_{18} VanGuard Pre-Column, 1.7 µm 2.1 mm x 5 mm (P/N 186003975)
Analytical column:	ACQUITY UPLC BEH C_{18}, 1.7 µm, 1 mm x 100 mm (P/N 186002346)
Column temp.:	0 °C
Mobile phase A:	water with 0.1% formic acid
Mobile phase B:	acetonitrile with 0.1 % formic acid
LC gradient:	2–40% of mobile phase B in seven minutes

SYNAPT G2-S*i* HDMS^E

Resolution mode:	ESI+
Capillary voltage:	3.0 kV
Sampling cone:	30 V
Source offset:	30 V
Source temp.:	80 °C
Desolvation temp.:	170 °C
Desolvation gas flow:	600 L/h
Scan time:	0.4 s

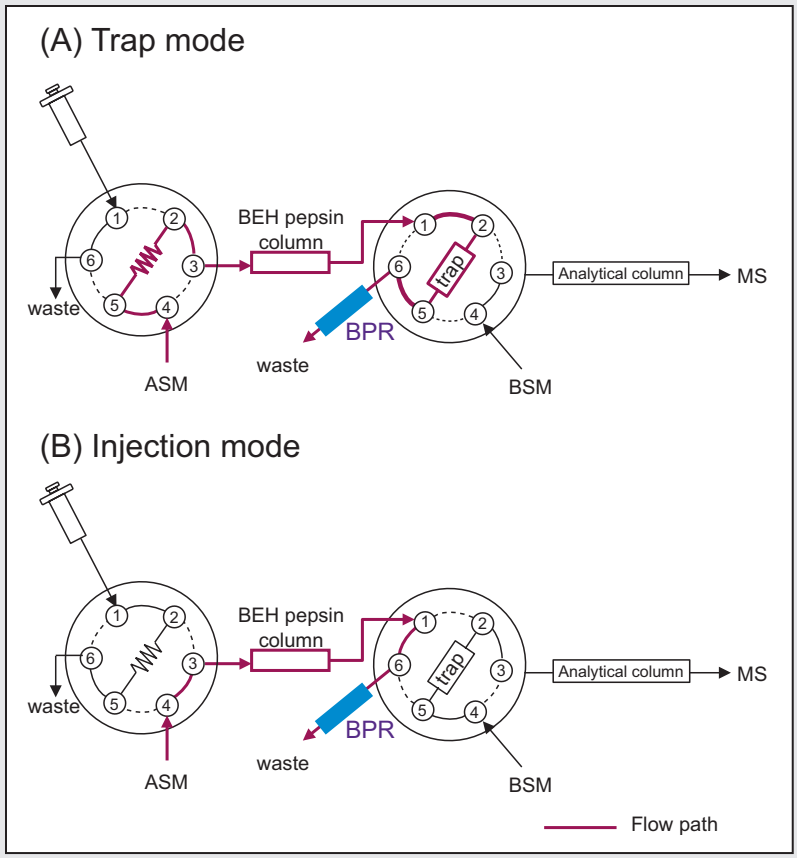

Figure 1. Fluidic schematic for the flow path in Trap (A) and Injection (B) modes. The red line indicates the flow path. The Back Pressure Regulator (BPR) is colored in blue. The auxiliary solvent manager (ASM) delivers mobile phase [0.1% formic acid in water, pH 2.5] for pepsin digestion. The binary solvent manager (BSM) drives the reversed-phase gradient [0.1% formic acid in water and 0.1% formic acid in ACN] for peptide trapping and separation.

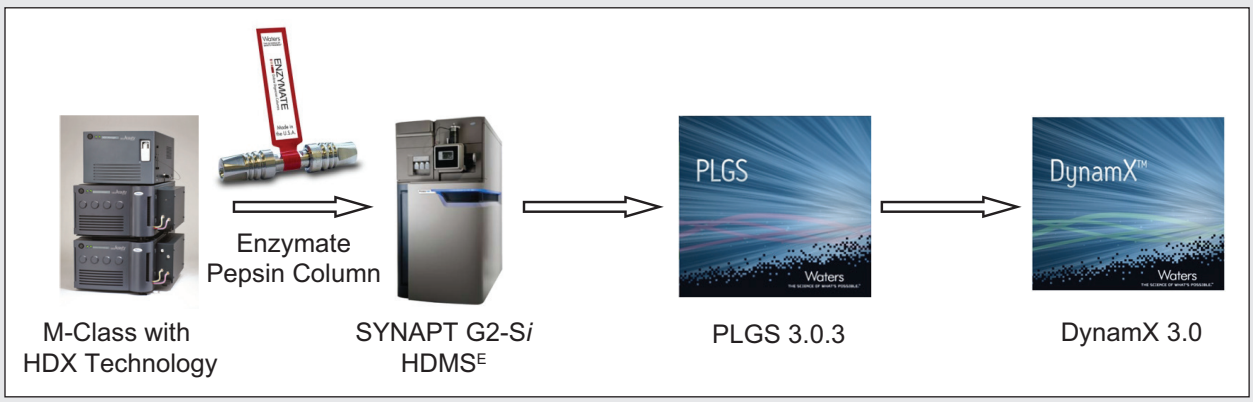

Figure 2. Waters integrated HDX solution.

RESULTS AND DISCUSSION

A pressurized online pepsin digestion was developed to run the HDX experiments, to improve sequence coverage results, and to enhance the spatial resolution. In this study, a systematical review of digestion conditions and their effects on digestion efficiency under higher pressure was performed. IgG2 – a protein with compact structure that is known to resist enzymatic digestions – was chosen as a model protein to display the procedure of pressurized digestion and to illustrate the effects of various conditions on digestion and back exchange. Key parameters that influence digestion efficiency during online pepsin digestion were investigated.

DIGESTION TEMPERATURE

The temperature of the pepsin column compartment inside the HDX manager was set at 0, 10, and 15 °C. Improved digestion efficiency (better sequence coverage and more overlapped peptides) was obtained for both heavy and light chain at higher temperature as shown in Figure 3A. 15 °C was chosen for the following experiments.

QUENCH HOLDING TIME

Quench holding time is the reaction time of both denaturation and reduction after quenching buffer, containing TCEP and GdnHCl, has been added. All the quench holding reactions were performed at 0 °C. As the quench holding time increased, so too did the digestion efficiency for IgG2 increase (Figure 3B).

DIGESTION AND DESALTING FLOW RATE

Higher flow rate and longer desalting time provide a cleaner MS background, especially for removing salts from the quench buffer. In the following experiments, pepsin digestion was performed at 100 µL/min for one minute and 200 µL/min for the remaining three minutes (Figure 3C).

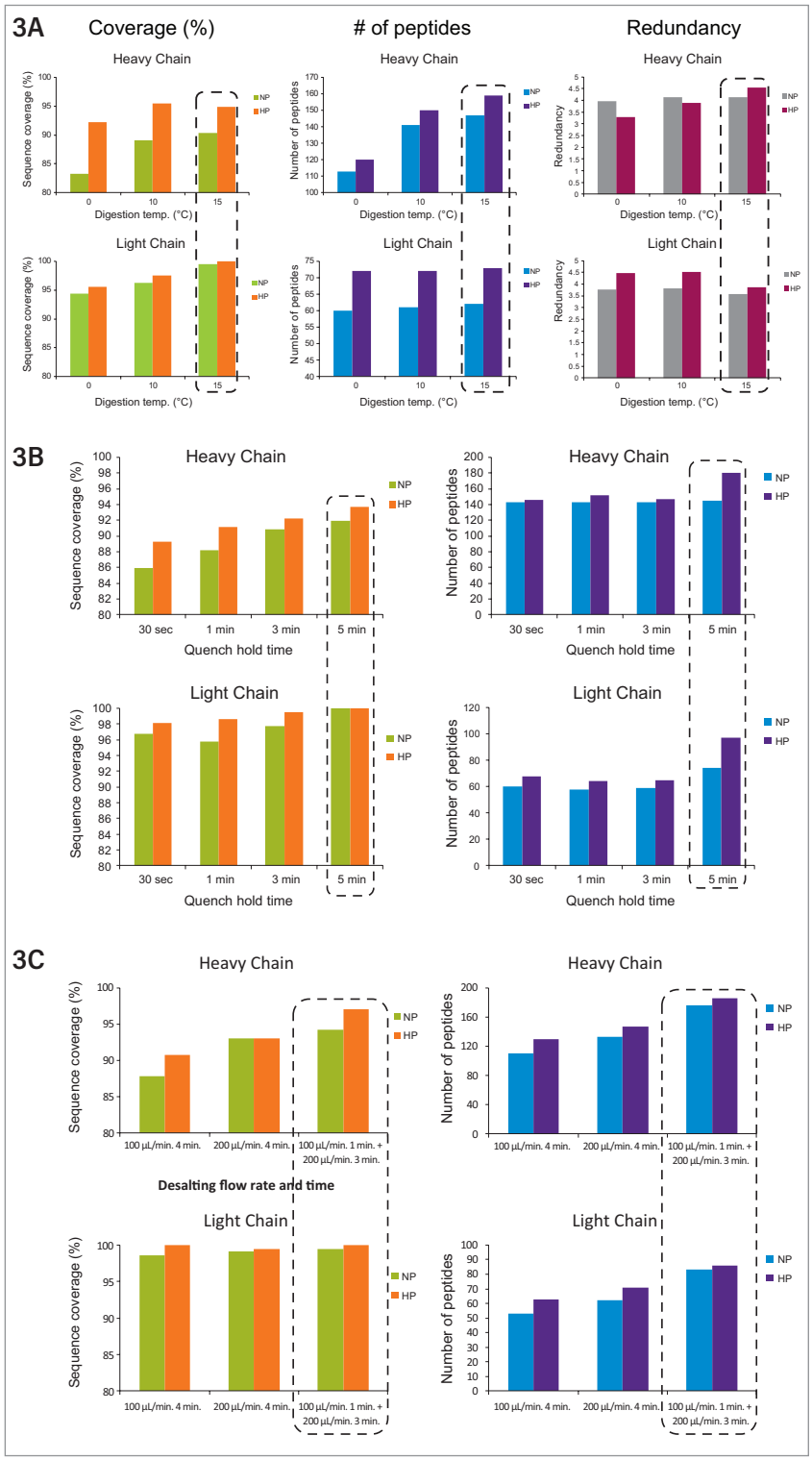

Figure 3. Sequence % coverage (left column) and # of peptides (middle column) of IgG2 (heavy chain: top; light chain: bottom) were determined and compared at different (A) digestion temperatures; (B) quench holding times; and (C) desalting/digestion flow rates under normal pressure (NP) and higher pressure (HP). The conditions that were chosen for the following experiments are highlighted in the dashed box.

THE CONCENTRATION OF CHAOTROPIC REAGENTS

Since pressure alone may not be sufficient enough to denature some stable proteins – particularly proteins crosslined with disulfide bridges – common reducing agents are used to help promote pressure-induced denaturation. Higher pressure presents opportunities to decrease the use of chaotropic reagents, which simplifies post-digestion and is more compatible with downstream MS analysis. Various concentrations of GdnHCl (from 2 to 4 M) and TCEP (from 0.2 to 0.5 M) were evaluated. Based on results (not shown here), comparable, or slightly better, digestion efficiency was obtained under high pressure using less TCEP (0.4 M) and GdnHCl (3 M), compared to the sample under normal pressure using more TCEP (0.5 M) and GdnHCl (4 M).

The combined, optimized conditions were as follows. The IgG2 samples were quenched through addition of pre-chilled quenching buffer that contained 0.4 M TCEP and 3 M GdnHCl. The sample was then held for five minutes at 0 °C. The digests passed through an Enzymate pepsin column at 100 µL/min flow in 0.1% formic acid mobile phase solvent for one minute, and desalted at 200 µL/min flow rate for three minutes. An online pepsin digestion was performed at 15 °C. A higher number of overlapped peptides with shorter lengths were generated by the Enzymate column under higher pressure (Figure 4). The protein sequence coverage is improved from 94% to 97% under pressurized digestion. Peptides that were unique to the pressurized digestion are shown in yellow.

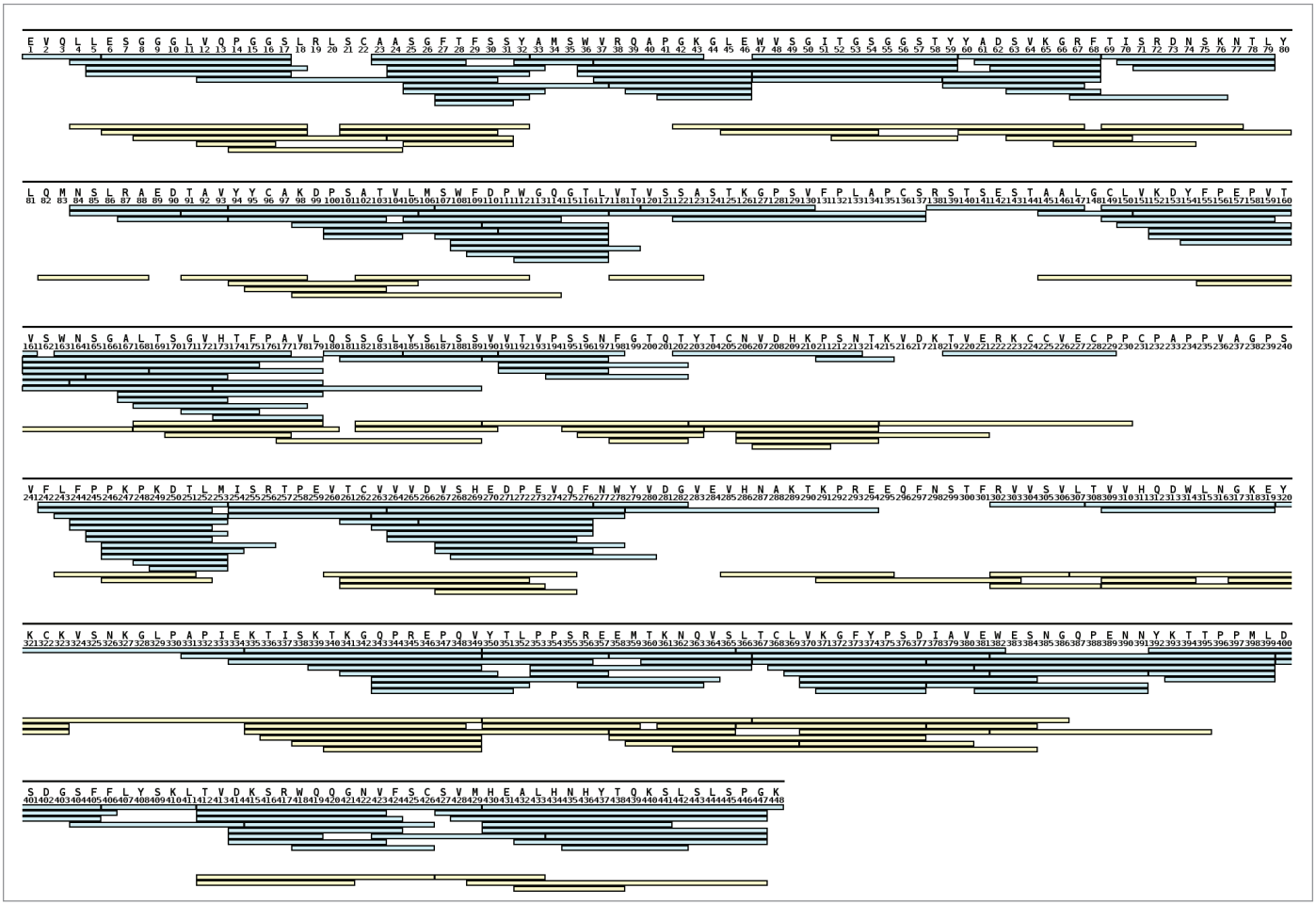

Figure 4. Sequence map of heavy chain of IgG2. The peptides identified under normal pressure are shown as cyan-colored bars. Unique peptides generated by high-pressure digestion are shown in yellow.

DIGESTION CONDITIONS IMPACT DEUTERIUM BACK EXCHANGE

Monitoring the deuterium loss of the analytical system is important in the HDX method; significant deuterium loss during digestion can be a problem when measuring deuterium uptake of peptides in HDX experiments. As expected, higher digestion temperature caused more deuterium loss. However, there was no significant difference in deuterium loss between 10 and 15 °C for both angiotensin II (DRVYIHPF) and bradykinin (RPPGFSPFR) peptides (Figure 5A). The back exchange caused by the mass spectrometer was also determined by directly infusing fully deuterated peptide mixture. ~13% and 15% of deuterium loss for bradykinin and angiotensin II was observed, respectively. Because the quench holding was performed at low pH and 0 °C, only a slight increase in deuterium loss was observed from 30 seconds to 5 minute quench holding time (Figure 5B). The online digestion time and desalting flow rate changed the reaction time of proteins and the immobilized enzyme; however, these factors had minimum impact on the back exchange. To obtain a relatively longer digestion time (~one minute for flow rate at 100 µL/min) and a cleaner MS background, 100 µL/min for the first minute and 200 µL/min for the remaining three minutes were chosen for the loading step of IgG2.

Based on these results, comparable levels of deuterium loss under pressure or non-pressure were obtained for fully deuterated angiotensin II and bradykinin. Representative mass spectra of bradykinin are shown in Figure 5D.

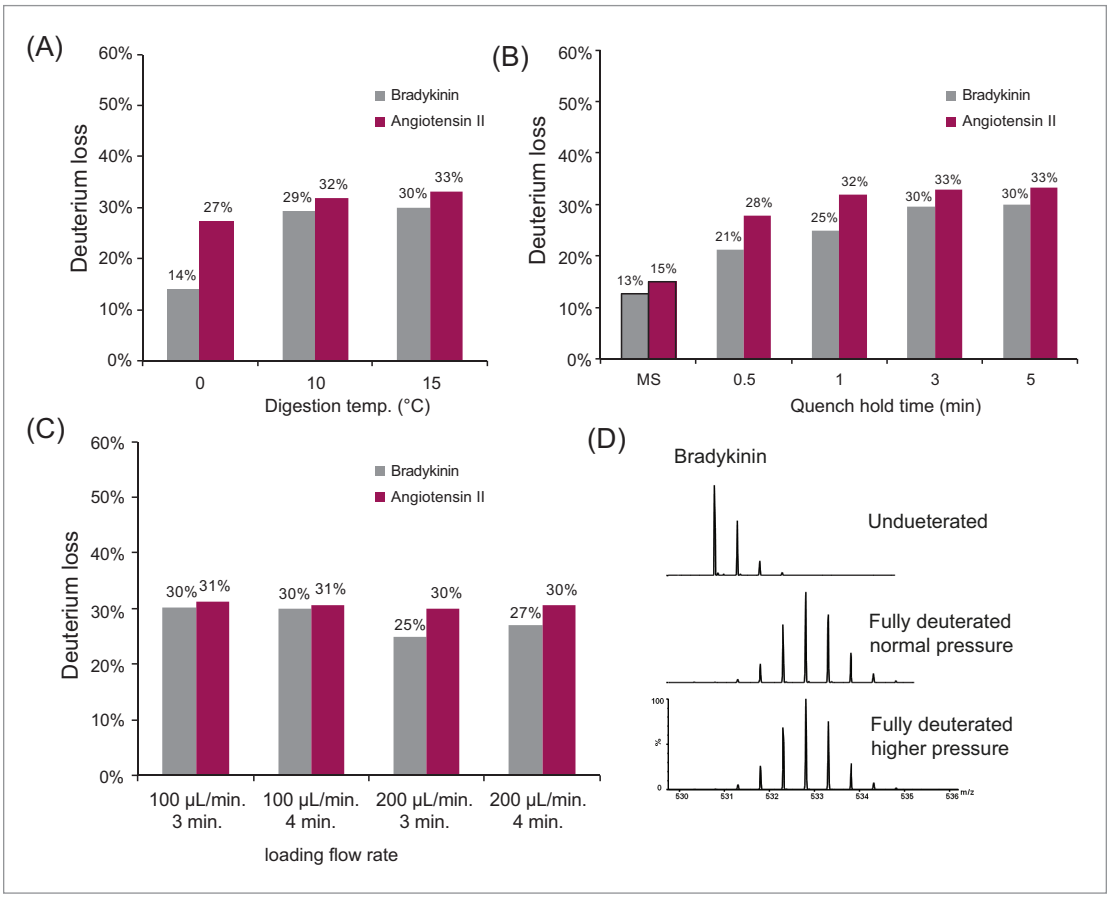

Figure 5. Comparison of deuterium loss from fully deuterated bradykinin and angiotensin II at (A) various digestion temperatures, (B) quench holding times, and (C) digestion/desalting flow rate. The deuterium loss caused by the mass spectrometer source is highlighted with a black border in 5B. Similar isotope profiles were found in both peptides under normal pressure and high pressure (5D). High pressure caused a slightly higher deuterium loss (<5%).

CONCLUSIONS

When used under high pressure, the Waters Enzymate Online Digestion column – a pepsin-immobilized BEH column – increased protein digestion efficiency to ~15,000 psi. Results from studying the model protein IgG2 showed an increase in overlapping peptides with shorter lengths under high-pressure digestion. This led to both increased protein sequence coverage and to higher spatial resolution when compared to normal pressure under the same digestion conditions. In addition, less chaotropic reagents were needed under high-pressure digestion to achieve similar digestion efficiency as normal-pressure digestion. The side benefit of using less salt is lesser MS background noise and improved overall LC-MS data.

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