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RESEARCH ARTICLE

Minimizing Carry-Over in an Online Pepsin Digestion System used for the H/D Exchange Mass Spectrometric Analysis of an IgG1 Monoclonal Antibody

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Abstract

Chromatographic carry-over can severely distort measurements of amide H/D exchange in proteins analyzed by LC/MS. In this work, we explored the origin of carry-over in the online digestion of an IgG1 monoclonal antibody using an immobilized pepsin column under quenched H/D exchange conditions (pH 2.5, 0 °C). From a consensus list of 169 different peptides consistently detected during digestion of this large, ~150 kDa protein, approximately 30 % of the peptic peptides exhibited carry-over. The majority of carry-over originates from the online digestion. Carry-over can be substantially decreased by washing the online digestion flow-path and pepsin column with two wash cocktails: [acetonitrile (5 %)/ isopropanol (5 %)/ acetic acid (20 %) in water] and [2 M guanidine hydrochloride in 100 mM phosphate buffer pH 2.5]. Extended use of this two-step washing procedure does not adversely affect the specificity or activity of the immobilized pepsin column. The results suggest that although the mechanism of carry-over appears to be chemical in nature, and not hydrodynamic, carry-over cannot be attributed to a single factor such as mass, abundance, pI, or hydrophobicity of the peptides.

Key words: H/D exchange mass spectrometry, Pepsin, Proteolysis, On-line digestion, Carry-over

Introduction

Over the past two decades, amide H/D exchange coupled with MS-based detection has matured into a valuable tool in the analysis of protein structure in solution [1–4]. When a protein is placed in a large excess of D_2O, the backbone amide hydrogens undergo exchange with deuterium at rates that are characteristic of protein conformation and dynamics. Mass analysis reveals the kinetics of deuterium incorporation, usually expressed in the form of a deuterium uptake curve. The technique is widely used to detect ligand binding [5] and to understand ligand-induced conformational changes [6], to define the effects of protein-protein interactions [7, 8], to understand the effects of post-translational modifications or other chemical changes [9], to characterize the dynamics of intrinsically disordered proteins [10, 11], to map protein–protein interfaces [12], and to evaluate therapeutic proteins as part of biopharmaceutical comparability studies [9].

Localized information, at a resolution of ~10 amino acid residues, can be achieved by subjecting deuterated proteins to proteolysis under slow-exchange conditions (so-called quenched conditions, pH ~2, 0 °C) [1, 13]. Acid-tolerant proteases such as pepsin are required for this digestion step. Even under quenched exchange conditions, the slow deuterium loss during digestion in H_2O-based solutions demands rapid proteolysis. For this reason early H/D-MS

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work employed high concentrations of solution-phase pepsin. The high protease concentration can interfere with the subsequent LC-MS analysis, and even at high concentrations, digestion is often inadequate. The development of online systems using immobilized pepsin columns for H/D-MS [14] greatly improved digestion efficiency and eliminated pepsin from the downstream analysis.

The abundance of the peptides generated by rapid proteolysis with immobilized protease columns can lead to carry-over artifacts. The problem of carry-over is particularly vexing in H/D exchange experiments because the retained peptide undergoes extensive back-exchange during the delay between sample injections. Such back-exchanged carry-over will manifest itself in the mass spectral data as isotopic profile doublets consisting of both a deuterated peptide feature and a corresponding undeuterated feature left over from the previous injection (for example, see Figure 4 in work of the Fang et al. [15]). Such carry-over can produce a mass spectral profile that can be mistaken for exchange via the EX1 mechanism [15]. In this same study, mitigation of carry-over of a collection of so-called sticky peptic peptides in reversed-phase column and trap of a UHPLC was systematically explored [15]. The potential for the immobilized pepsin column to also contribute to carry-over artifacts in H/D exchange MS experiments was suggested but not investigated by Fang et al., presumably since the wash protocols identified in their work (for minimizing carry-over in the reversed-phase column and trap) were not expected to be compatible with maintaining pepsin's enzymatic activity.

In the course of recent method development for the H/D-MS analysis of an IgG1 monoclonal antibody in our laboratories, we encountered excessive carry-over of peptic peptides in our online digestion system. Given the recent interest in the potential utility of hydrogen/deuterium exchange mass spectrometry for analysis of IgG monoclonal antibodies (mAbs) [9, 16–18], it is important to assess the nature and extent of peptide carry-over issues with each mAb, especially given the large number of peptides generated with these ~150 kDa proteins. In this paper, we show that the primary source for carry-over for our system arose not from the reversed-phase columns, but from the online pepsin digestion process. We further demonstrate that a simple two-step washing procedure can be used to substantially decrease this carry-over and that 100 cycles of the washing process does not adversely affect the activity and specificity of the immobilized pepsin column. Since previous H/D-MS studies with mAbs have not mentioned this aspect of method development, this work serves as a case study to raise awareness and provide a protocol that minimized carryover during H/D-MS studies with a particular IgG1 mAb.

Experimental

Reagents and Materials

LC mobile phases were prepared from LC/MS grade acetonitrile and water (Fisher Scientific, Fair Lawn, NJ) and formic acid (Thermo Scientific, Rockford, IL, 99+ % LC/MS grade). Phosphoric acid solution and acetic acid (both HPLC grade, >98.5 %) were obtained from Fluka (St Louis, MO). Isopropanol (Optima LC/MS grade) was obtained from Fisher Scientific. Guanidine hydrochloride, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), porcine pepsin were obtained from Sigma-Aldrich, St Louis, MO. The IgG1 monoclonal antibody stock contained 50 mg mL^{-1} protein containing 0.005 % polysorbate 80 in 10 mM sodium-histidine buffer at pH 6.

Sample Preparation

The antibody sample was diluted to 10 mgmL^{-1} working concentration with 10 mM sodium histidine buffer at pH 6.0. For a typical mAb digest run, 5 µL of antibody sample at working concentration was diluted with 95 µL of 0.1 % formic acid. This solution was mixed with a reducing quench buffer containing 500 mM TCEP, 4 M guanidine hydrochloride, and 200 mM sodium phosphate buffer at pH 2.5 in a 1:1 ratio by volume. The antibody sample was incubated at 0 °C for one min for disulfide bond reduction and then 10 µL of this solution (approximately 16 pmol of antibody) was injected into sample loop of the LC. The short incubation time, low temperature, and low pH mimic typical quenching conditions for H/D exchange.

Chromatography and Mass Spectrometry

A single-valve, two-pump system for H/D exchange with a refrigerated valve and column compartment was used to maintain 0 °C throughout the online digestion and separation, as described previously (see Figure 1) [19]. Following sample injection, a loading pump carried the sample through a 50 mm × 2.1 mm immobilized pepsin column, prepared in-house [14], using 0.1 % formic acid at a flow rate of 200 µL min^{-1}. The resultant peptides were captured and desalted on a reversed-phase trap (Peptide Concentration and Desalting Microtrap; Bruker-Michrom, Auburn, CA, USA) for 5 min at the same flow rate. For gradient elution the mobile phases were 0.1 % formic acid (A) and 90 % acetonitrile/10 % water/0.1 % formic acid (B). The peptides were eluted from the trap and separated on a revered-phase analytical column (ZORBAX 300SB-C18, 50 mm × 1 mm, 3.5 µm particle diameter; Agilent Technologies, Santa Clara, CA, USA) using a 15–40% B gradient over 5 min. Following the shallow gradient, the trap and column were cleaned using a 40–90% B gradient followed by a 5–90% B gradient in 1 min each with 1 min of equilibration at the highest and lowest organic content. The needle port and sample loop were cleaned between injections by overfilling with mobile phase B followed by mobile phase A. Following positive electrospray ionization (3.5 kV capillary), mass spectra were acquired with a time of flight mass spectrometer (model 6220; Agilent Technologies) operating in 2 GHz extended dynamic range mode with the fragmentor set to 150 V. The

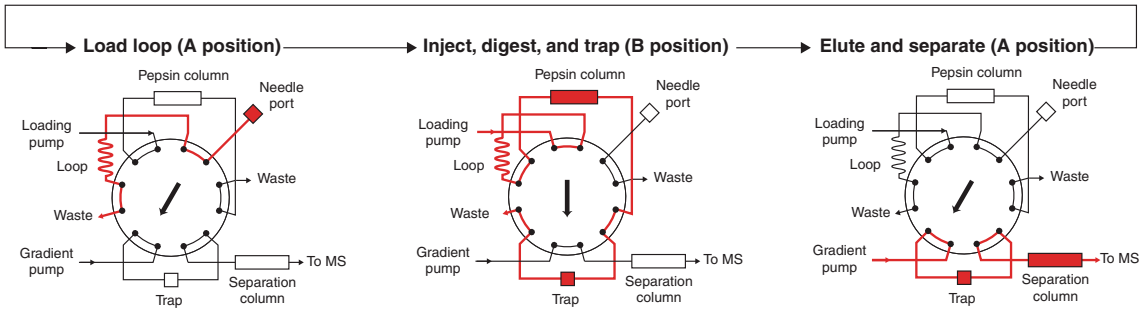

Fig. 1. Flow diagram of the LC/MS system for online digestion with an immobilized pepsin column used for carry-over studies. The system is comprised of a two-position, 12 port valve [19]. The red regions of the flow path denote the location of sample at the different stages of analysis. In the first step, sample is loaded through the needle port with the valve in the A position. In the second step, the valve is switched to the B position. The loading pump carries sample through the pepsin column where the resulting peptides are captured by the reversed-phase peptide trap. In the third step, the valve is switched back to the A position and a water-acetonitrile gradient elutes the peptides from the trap onto a reversed-phase column for separation and then to the mass spectrometer for analysis

desolvation gas flow was 10 L min^{-1} at 325 °C with a nebulizer pressure of 20 psig.

Following the completion of a protein digest run, blanks were run to evaluate carry-over. An LC blank was run first to evaluate carry-over in the peptide trap and the reversed phase column. For the LC blank, the valve was kept in the load position (see Figure 1); there was no injection of the contents of the loop. One or two digestion blanks followed the LC blank. For the digestion blanks, 10 µL of 10 mM sodium histidine buffer diluted 1:1 with the reducing quench buffer was injected. The same gradient was used for all blanks and digests.

Peptide Identification

Preliminary peptide identities were assigned on the basis of accurate mass (±20 ppm) using the known antibody sequence. Peptides that could not be unambiguously assigned by mass mapping were assigned in separate MS/MS measurements. The antibody sample was diluted with quench buffer as described in the *Sample Preparation* section. The sample was infused through an immobilized pepsin column at 200 µL min^{-1} at 4 °C using a syringe pump. The eluate was collected and flash frozen with liquid nitrogen. The resulting peptide mixture was then analyzed by tandem mass spectrometry by a linear quadrupole ion trap mass spectrometer (LTQ-XL; Thermo Scientific). The peptides were separated by HPLC (Shimadzu, Columbia, MD) through a reversed-phase C18 column (ZORBAX SB300-C18, 2.1×100 mm, Agilent) with a 120 min 0–60 % B gradient (A: 99 % H_2O, 1 % ACN, 0.1 % formic acid; B: 99 % ACN, 1 % H_2O, 0.1 % formic acid; 200 µL min^{-1} flow rate). The capillary temperature and voltage were set to 275 °C and 48 V, respectively. The source current was set to 100 µA, the tube lens was set to 115 V, and the sheath gas flow rate was set to 40. Data-dependent MS/MS analysis was performed using the Xcalibur 2.0 software (Thermo Scientific). Survey mass spectra were acquired in the LTQ over an *m/z* range of 300–2000. The three most intense ions in each spectrum were selected for fragmentation by collision-induced dissociation using normalized collision energy of 35 V. Ion selection threshold was 1000 counts and the dynamic exclusion duration was 30 s. After the initial MS/MS run, inclusion and exclusion mass lists were included during additional runs to increase the number of confirmed peptides from the consensus list. Raw data were processed using the Proteome Discoverer 1.3 software (Thermo Scientific). Only MS/MS spectra with XCorr score of ≥2 were used to validate peptide assignments.

Pepsin Column Washing

The pepsin column wash process consisted of two injections of 100 µL of wash cocktail. Wash cocktail 1 was 5 % acetonitrile/5 % isopropanol/20 % acetic acid in water and wash cocktail 2 was 2 M guanidine hydrochloride/100 mM sodium phosphate at pH 2.5. Three different washing processes were evaluated: 2×wash cocktail 1, 2×wash cocktail 2, and wash cocktail 1+wash cocktail 2. The loading pump was used to carry each injection through the pepsin column and trap at a flow rate of 200 µL min^{-1} for 7.5 min (see Figure 1, middle panel). The injection port and sample loop were flushed between injections as described in the previous section.

Pepsin Column Stress Test

To determine if the pepsin column remained stable after extensive washing, an immobilized pepsin column was subjected to 200 wash injections, alternating between wash cocktails 1 and 2, for a total of 100 cycles of the two-step washing process. This test was performed using an HDX PAL robot (LEAP Technologies, Carrboro, NC, USA) with a refrigerated compartment held at 0 °C housing the trap, columns and valves. The HDX PAL system is comprised of two valves and features a back-flush of the pepsin column to

waste during the gradient elution step. In other respects, the configuration of the pumps and columns is the same. The pepsin column stress test began with an online antibody digest run. The digest run was followed by 100 wash cycles, as described above. After the 100 wash cycles, the online digestion of the antibody was repeated.

Data Processing

Peptide mass spectral features from digestions were extracted using the Find by Molecular Feature algorithm in MassHunter Qualitative Analysis with BioConfirm (Agilent Technologies). The peptides were quantified using the MS intensity from spectra averaged across the chromatographic band. Peptides were initially assigned by mapping them onto the antibody sequence by mass-matching (±10 ppm) using Protein Prospector (MS-Product, http://prospector.ucsf.edu). An exclusion list, developed from online digest blanks run before antibody digestion, was used to eliminate pepsin autodigestion peptides. The list of extracted antibody peptides was filtered to exclude peptides with low MS response (<3000 counts) and to remove peptides with fewer than five residues or more than 25 residues. To account for variability across the digest runs obtained on different days, a consensus list of 169 peptides found in all digest runs (Figures 2, 3, and pre-stress digest of Figure 4) was constructed. Consensus peptides were those peptides that matched mass within ±10 ppm across all runs and had a retention time standard deviation of less than 0.2 min. Since the pepsin column stress test was run using a different LC system, only the 10 ppm mass match criterion was imposed for the pre-stress list. For carry-over detection, a signal threshold of 750 counts, the approximate chemical noise level, was imposed. 101 of the 169 consensus peptide assignments were confirmed using MS/MS; 57 of which were not carried-over and 44 of which were carried over in blank digestions. The pI and hydrophobicity for each identified peptide was determined using *ProtParam* [20, 21] on the ExPASy server (http://web.expasy.org/protparam/).

Results and Discussion

Because carry-over can introduce serious artifacts into H/D exchange measurements, we routinely undertake a carry-over study at the beginning of LC method development for H/D exchange studies of proteins. Recently, method development work in our laboratories with an IgG1 monoclonal antibody revealed substantial carry-over in blanks following online pepsin digestion of the protein. Some level of carry-over can usually be found in LC/MS data if one looks hard enough. For quantitative analysis, rigorous definitions of carry-over can be derived that can be used to set thresholds for identifying significant carry-over effects [22, 23]. In the case of qualitative analyses, such as proteomic studies, however, any amount of carry-over can potentially result in false positives [24]. In H/D-MS, carry-over can produce a so-called false EX1 MS signature that can skew the average mass determination [15].

Online Digestion is the Major Source of Peptide Carry-Over

The flow path for our online digestion system [19] at different stages in the analysis process is shown in Figure 1. The system consists of a two-position 12-port valve. In the A position, the loop is loaded with sample, the immobilized pepsin column is in-line with the loading pump, and the reversed-phase trap and separation column are in-line with the gradient pump. Switching to the B position puts the loop and peptide trap in-line with the pepsin column and loading pump (0.1 % formic acid). During this stage, the contents of the loop are carried through the pepsin column and then pass through the trap. Following a suitable digestion interval, the valve is switched back to the A position. In this position, the reversed-phase trap and separation columns are in-line with the gradient pump; a water-acetonitrile gradient is then used to elute and separate the peptides.

Representative chromatographic and mass spectral data illustrating the carry-over problem are shown in Figure 2a and b, respectively. The top panels (labeled mAb digest) show a well-defined chromatographic peak in the extracted ion chromatogram for a selected mAb peptide. To isolate sources of the carry-over in the chromatographic system, we used a series of blank runs following an antibody digestion run. First, an LC blank followed each injection of the antibody for online digestion. The LC blank was simply a second gradient cycle run while the valve was held in the A position (see Figure 1); the reversed-phase trap and separation column were not re-exposed to eluate from the immobilized pepsin column. The second set of panels in Figure 2a and b (labeled LC blank) show a minimal MS response for the peptide (<1 % carry-over, quantified by the MS intensity). As the next set of panels show, however, the peptide was readily detected in a subsequent blank digestion (an injection of sample buffer without protein) with a mass spectral response 26 % of its original value. Hence, the major source of carry-over for this peptide was the immobilized pepsin column, not the reversed-phase trap and column.

To account for the inherent variability in digestion across the numerous LC/MS runs in this study, a consensus list of 169 peptides found in common across all digests was employed. For this reason, the reported number of peptides detected in the antibody digests is always 169. The number of consensus peptides detected in the antibody digest and in the blanks are shown in Figure 2c. For the LC blank, out of 169 peptides detected in the digestion of the antibody, only 8±5 peptides were carried over. This result shows that only ~5 % of the peptides could be detected as carry-over in the trap and the reversed phase column. On average, the

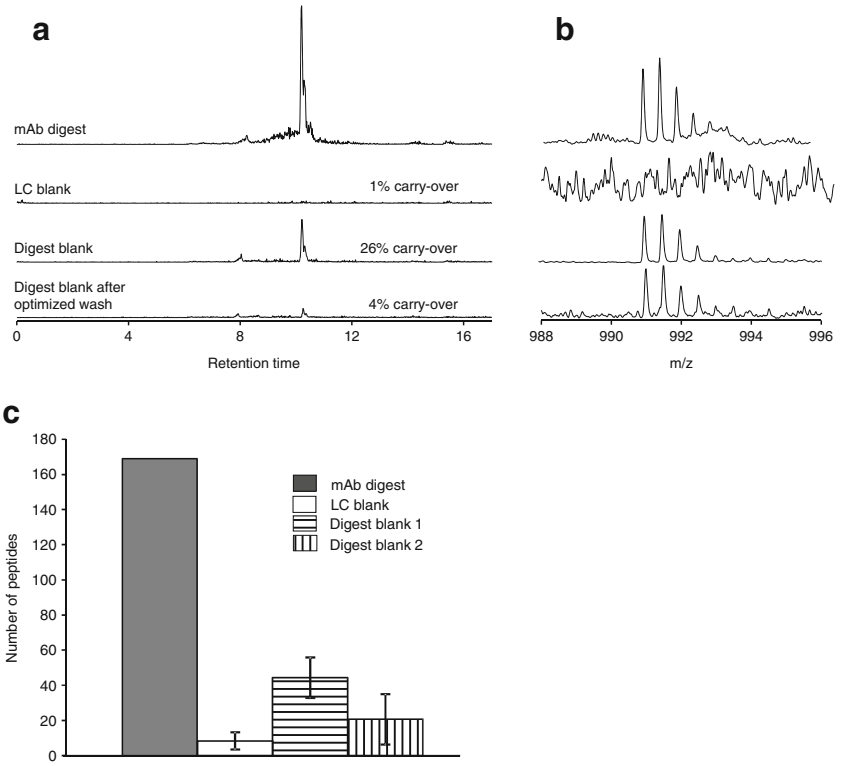

Fig. 2. Carry-over from the online digestion of an IgG1 monoclonal antibody with an immobilized pepsin column arises from online digestion. Extracted ion chromatograms (**a**) and mass spectra (**b**) of the peptide PEVTCVVVDVSHEDPEVK show that carry-over of this peptide was insignificant in the LC blank compared with the carry-over in the digestion blank. A digest blank following a pepsin column wash (see text for details) shows that washing eliminated the observed carry-over. (**c**) A list of 169 consensus peptides found in all digestion runs was used to identify carried-over peptides. Only a small number of these consensus peptides were found in the LC blank while a much larger number were detected in two subsequent digestion blanks. Based on flow diagram (see Figure 1), the online pepsin digestion is thus the major source of peptide carry-over. The error bars denote one standard deviation for three independent replicates (since all 169 peptides were found in all digestions, no error bars are shown for the digest)

peptides carried-over in the LC blank had an MS intensity of 3.4 % of their intensities in the digest. In contrast, blank digestions, showed significantly more carry-over (44±12 peptides) that persisted through a second blank digest (21± 14 peptides) and the average intensity was 9 %. These results show that most of the carry-over from this antibody digestion arose from the immobilized pepsin digestion process, not from peptides retained on the reversed-phase column and trap. Similar results were obtained when these studies were performed using both a different in-house column and a commercial column (Applied Biosystems, Foster City, CA) (data not shown). We have also observed that the number of carried-over peptides increased as the mAb load was increased (data not shown).

Pepsin Column Washing Decreases Peptide Carry-Over

Having established that online digestion was the major source of carry-over, we developed a pepsin column wash procedure. A wide variety of washing reagents and washing cocktails to mitigate peptide carry-over have been described in the literature (for example [15, 24]) for the reversed-phase column and trap. In the case of the online pepsin column, however, it is essential that the washing reagents do not irreversibly perturb the immobilized enzyme. The manufacturer's literature indicated that immobilized pepsin would tolerate low concentrations of organic co-solvents and moderate concentrations of denaturants. We thus evaluated two different wash solutions: "wash 1" consisted of acetonitrile (5 vol%), isopropanol (5 vol%), acetic acid (20 vol%) in water; and "wash 2" contained 2 M guanidine hydrochloride and 100 mM sodium phosphate buffer at pH 2.5. Two sequential loop volumes of the wash cocktails were injected onto the pepsin column (see Figure 1). Figure 2a and b (bottom panels) show that there was a significant decrease in MS response for the representative peptide in both the mass spectrum and the corresponding extracted ion chromatogram (~4 % carry-over). The effects of different combinations of the two washes on peptide carry-over in digestion blanks are shown in Figure 3. For example, wash 1 +wash 2 was equally effective at minimizing carry-over as

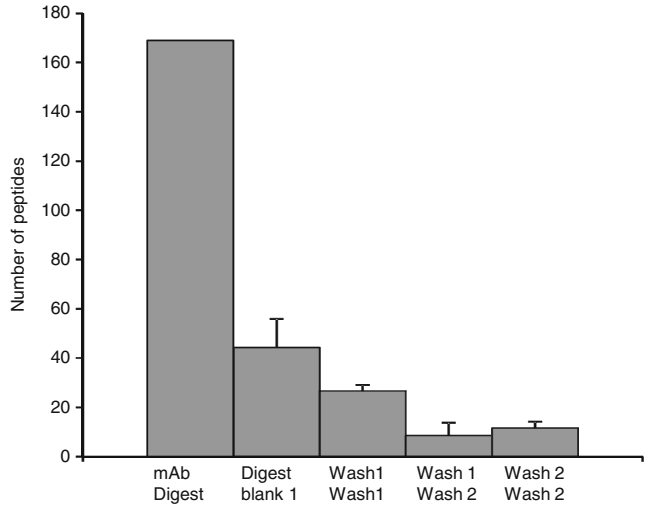

Fig. 3. Injections of different combinations of two wash cocktails decreased the number of consensus peptides (see Figure 2c) detected as carry-over in the digestion blanks. For comparison, the mAb digest and digest blank 1 values are duplicated from Figure 2c. For the composition of the wash cocktails, see the Methods section. The error bars denote one standard deviation for three independent replicates (since all 169 peptides were found in all digestions, no error bars are shown for the digest)

2×wash 2 (9±5 and 12±3 carried-over peptides, respectively). In contrast, injection of two loop volumes of wash 1 was significantly less effective than the other two combinations (26±3 carried-over peptides), yet still decreased carry-over in the digest blank relative to carry-over observed without washing (44±12 carried-over peptides). These results demonstrate that for this IgG1 mAb, peptide carry-over can be substantially decreased by post-digest injection of the wash cocktails through the online immobilized pepsin column.

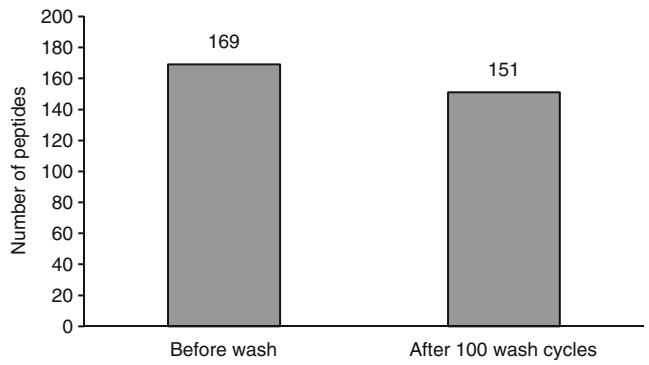

Fig. 4. A pepsin column stress test consisting of 200 alternating injections of wash cocktails 1 and 2 did not cause a substantial decrease in the number of detected consensus peptides (see Figure 2c) in the online digestion of an IgG1 monoclonal antibody

Pepsin Column Washing Does Not Degrade Pepsin Column Performance

To determine if the washes would have a detrimental effect on the performance of the pepsin column, we subjected a pepsin column to a stress test consisting of 200 alternating injections of wash 1 and wash 2. The pepsin column was used to digest the antibody before and after the stress test. After the stress test, 151 of the 169 peptides were detected (Figure 4), indicating that the pepsin column retains most of its specificity. To determine if enzymatic activity was lost, we compared the MS response of each of the 151 individual peptides before and after the stress test. Figure 5 shows a plot of peptide MS response after the stress test versus MS response before the stress test. The figure shows a near-unity slope (0.97) with an excellent linear correlation (r^2=0.92), indicating that there was little loss in peptide abundance following the pepsin column stress test. To further examine the effects of wash protocols on pepsin activity, we examined the mass distribution of all abundant peptides that could be mapped onto the mAb sequence (see Table 1). The average peptide mass and standard deviations of the distributions were essentially the same before and after the stress test (1697±1250 Da versus 1769±1366 Da), although there was an 18 % decrease in the total number of peptides (this analysis relies on peptide mass rather than length, since not all peptide assignments in this expanded list of peptides were confirmed by MS/MS). The results indicate that the stress test had little effect on the selectivity of the immobilized pepsin. Taken together, these results demonstrate

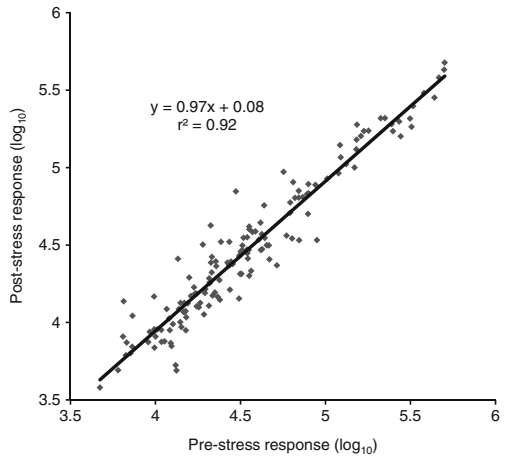

Fig. 5. A pepsin column stress test consisting of 200 alternating injections of wash cocktails 1 and 2 did not cause a substantial decrease in the MS response of the consensus peptides. Each point corresponds to one of the 151 consensus peptides detected after the pepsin column stress test (see Figure 4)

Table 1. Size Distribution and Number of IgG1 mAb Peptic Peptides[a] Before and After Stress Test of Immobilized Pepsin Column. Stress Test Consisted of 200 Alternating Injections of Wash 1 and Wash 2; see text)

	Before stress test	After stress test
n	565	461
Mean mass (Da)	1769	1697
Median mass (Da)	1312	1292
Standard deviation (Da)	1367	1249

[a]MS features that mapped onto the mAb sequence with better than ±10 ppm mass accuracy and had an MS base-peak response greater than 10^4 cps at the chromatographic peak maximum

that extensive washing of the pepsin column does not lead to a pronounced decrease in either specificity or activity.

Basis of the Carry-Over

A variety of hydrodynamic and chemical effects can cause carry-over [24]. Regions of unswept volume caused by tubing diameter mismatches, scratches in valve components, or inadequate flushing of a needle port are examples of mechanical sources of carry-over. In the experimental setup employed here, the digestion flow-path was flushed continuously by the loading pump (see Figure 1) for 12 min during the gradient step and an additional 17 min during the LC blank shown in Figure 2. Despite this long washout period, significant carry-over persisted, as shown in Figure 2. In addition, the needle port was thoroughly flushed between injections. Finally, only a limited sub-set of the peptic peptides from the antibody digest were found to carry over. Taken together, these observations suggest that the observed carry-over is not attributable to hydrodynamic effects, rather it appears to be chemical in nature. If undigested protein were carried over, then all peptides would exhibit the effect. Thus, we conclude that it is carry-over of peptides or partially digested protein, rather than intact protein.

The observed carry-over has been shown to be primarily due to the immobilized pepsin column digestion process (Figures 2 and 3). Carry-over could potentially arise from the binding to the immobilized pepsin itself, the column stationary phase substrate, or chromatographic system components (frits, tubing, etc.). According to the manufacturer's literature, the POROS substrate for pepsin immobilization is a cross-linked poly(styrene-divinylbenzene). Carry-over could therefore arise from slow release of hydrophobic or aromatic peptides from the stationary phase. Carry-over could also potentially arise from specific binding between pepsin and certain amino acid sequences found in the antibody. For example, pepsin inhibitor-3 (PI-3) from *Ascaris suum* is a protein that reversibly inhibits pepsin through the tight binding of short linear segment into the pepsin active site [25]. Alternatively, carry-over could also arise from nonspecific associations with the pepsin.

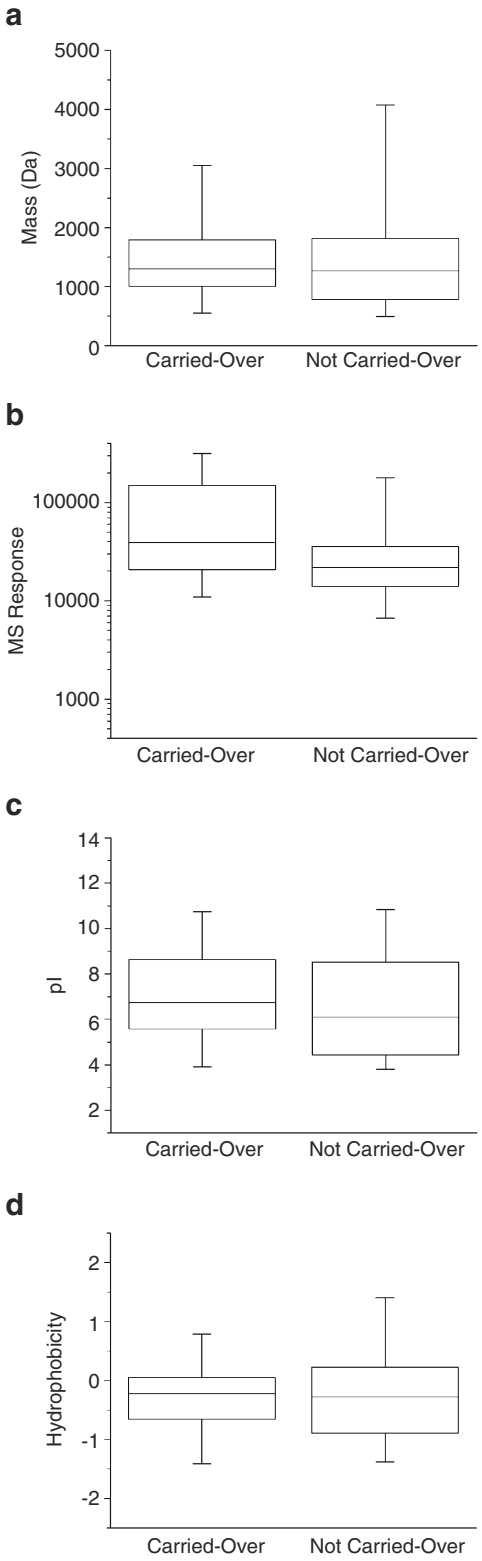

Fig. 6. Carried-over peptides are indistinguishable from peptides that were not carried over on the basis of (**a**) mass, (**b**) MS response, (**c**) pI, and (**d**) hydrophobicity [20]. In (**d**), negative values are hydrophilic and positive values are hydrophobic. For each category, the median value is designated by the horizontal band in the middle of the box; the box limits indicate the 25th and 75th percentiles. The bars denote the minimum and maximum values

To determine if there were any chemical characteristics that could distinguish between peptides exhibiting carry-over from peptides that were not carried over, we compared the two sets of peptides with respect to mass, MS response, pI, and hydrophobicity [20] (see Figure 6). We are unable to distinguish between peptides that were carried over from peptides that were not carried over on the basis of mass, pI, or hydrophobicity. This result suggests that there may in fact be several different mechanisms that contribute to carry-over. Our results suggest that carried-over peptides have a greater MS response, but this is attributable to carry-over becoming undetectable in peptides that had low MS response in the digest.

Conclusions

In this paper, we have demonstrated that significant amounts of carry-over can arise from the digestion of an IgG1 monoclonal antibody with an immobilized pepsin column under conditions used in H/D exchange mass spectrometry measurements. Further, we have shown that the online digestion itself was the major source of the observed carry-over. It remains to be determined whether similar carry-over would arise from other proteases [26–28] or whether similar results will be observed with different monoclonal antibodies (e.g., IgG2, IgG4). We have developed a two-step washing process for the immobilized pepsin column that greatly diminished the amount of carry-over for the IgG1 mAb being examined in our laboratory. Finally, we have shown with a pepsin column stress test that extensive washing (100 cycles) does not adversely affect either the specificity or activity of the pepsin column. Based on our analysis of chemical properties of the carried over peptides, there was no single distinctive property of the peptic peptides that can simply explain the basis for the observed carry-over.

The immobilized pepsin column washing procedure described in this work is not easily implemented on single valve systems [19] because injection interrupts the elution step (see Figure 1). This setup requires that the pepsin column wash must be delayed until the end of the gradient run. In contrast, with two-valve in-line systems [14], such as the robotic system we used for the pepsin column stress test, the wash process can be run in parallel with the elution and reversed-phase separation of the peptides.

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