

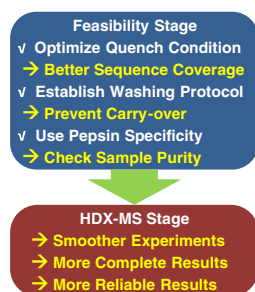
Optimization of Feasibility Stage for Hydrogen/Deuterium Exchange Mass Spectrometry

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Abstract. The practice of HDX-MS remains somewhat difficult, not only for newcomers but also for veterans, despite its increasing popularity. While a typical HDX-MS project starts with a feasibility stage where the experimental conditions are optimized and the peptide map is generated prior to the HDX study stage, the literature usually reports only the HDX study stage. In this protocol, we describe a few considerations for the initial feasibility stage, more specifically, how to optimize quench conditions, how to tackle the carryover issue, and how to apply the pepsin specificity rule. Two sets of quench conditions are described depending on the presence of disulfide bonds to facilitate the quench condition optimization process. Four protocols are outlined to minimize carryover during the feasibility stage: (1)

addition of a detergent to the quench buffer, (2) injection of a detergent or chaotrope to the protease column after each sample injection, (3) back-flushing of the trap column and the analytical column with a new plumbing configuration, and (4) use of PEEK (or PEEK coated) frits instead of stainless steel frits for the columns. The application of the pepsin specificity rule after peptide map generation and not before peptide map generation is suggested. The rule can be used not only to remove falsely identified peptides, but also to check the sample purity. A well-optimized HDX-MS feasibility stage makes subsequent HDX study stage smoother and the resulting HDX data more reliable.

Keywords: Carryover, Detergent, Digestion, Fos-choline-12, Fos-choline-14, Pepsin, HDX-MS, Hydrogen/deuterium exchange, Mass spectrometry, TCEP

Abbreviations: β -OG, n-octyl- β -D-glucopyranoside; C12E8, n-octaethylene glycol mono-n-decyl ether; CMC, critical micelle concentration; CYFOS-6, 6-cyclohexyl-1-hexylphosphocholine; DDM, n-dodecyl- β -D-maltopyranoside; Fos-choline-12, n-dodecylphosphocholine; GuHCl, guanidine hydrochloride; HDX, hydrogen/deuterium exchange; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PEEK, polyether ether ketone; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; TFA, trifluoroacetic acid

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Introduction

Bottom-up hydrogen/deuterium exchange mass spectrometry (HDX-MS) has become an essential tool in protein science [1–5]. The common approach to HDX-MS, which combines hydrogen/deuterium exchange (HDX) with online proteolysis, liquid chromatography (LC) separation and mass spectrometry (MS) analysis, was primarily used for basic research to understand biological systems in academia starting in the 1990s [6–11]. The pharmaceutical industry recognized the utility of the technology and ExSAR, the first contract research

organization offering HDX-MS service, opened its laboratory in 2002 to meet this need [12]. Up until the mid-2000s the primary utility of HDX-MS in the industry was investigation of protein–small molecule interactions to help small molecule drug programs [13–18]. From the late-2000s the majority of HDX-MS service trended towards epitope mapping [19–30], to meet the booming popularity of antibody drugs. More recently, in the 2010s, higher order structure analysis of therapeutic proteins, including comparability studies for biosimilar, is increasing its popularity [31–35].

Although the number of scientists practicing HDX-MS and the number of papers published on the topic have been growing every year [4, 36, 37], the practice of HDX-MS remains somewhat difficult [38]. One of the reasons is that HDX-MS

is a rather integrated technology. The technology includes automation of liquid handling [12, 13], online proteolysis [9, 39], liquid chromatography, mass spectrometry, and data analysis that requires two types of software (peptide search engine, such as Sequest [40] or Mascot [41], and centroid extracting software [42–45] such as HDExaminer or HDX Workbench). The HDX-MS practitioner must understand each component to at least a certain degree.

Another reason for its difficulty is the lack of information on the feasibility stage in the literature [46], although there are a few papers describing how to improve the resolution [47, 48] and how to minimize back exchange [49, 50]. After receiving (or producing) samples, an HDX-MS practitioner starts the feasibility study. In the feasibility stage, a practitioner optimizes digestion and LC-MS conditions to generate a set of peptic fragments that can provide a sequence coverage and resolution sufficient for the purpose of a project. The generation of a satisfactory peptide map in the feasibility stage triggers the initiation of the HDX study stage. However, when the practitioner reports the results of the HDX study, the feasibility stage results are usually not reported, because they do not have direct influence on the HDX study results.

The lack of information on the feasibility stage in the literature not only makes HDX-MS a more difficult technique, but also potentially requires unnecessary redundant efforts among the practitioners. Because the publications usually do not report the feasibility stage, the reasons why the digestion and LC-MS conditions were used for a certain HDX study is often not apparent. In the same way, reasons why other conditions were not applicable for a certain HDX study are also usually not discussed.

In this protocol, we discuss several considerations for the feasibility stage of HDX-MS analysis. More specifically, we describe how to optimize quench conditions, how to eliminate carryover, and how to utilize the pepsin specificity rule.

Experimental

Sample Preparation for Feasibility Stage

Typically the total volume of a protein sample and an aqueous buffer (which is replaced with deuterated buffer in the subsequent HDX stage) in the system is 20 μL . The ratio of the protein sample and the aqueous buffer varies between 1:1 and 1:9, depending on the concentration of the protein sample. The solution is then added a 30 μL of chilled (0 $^{\circ}\text{C}$) quench buffer, mixed, and immediately injected to the automated system described below for analysis.

General Process for Feasibility Stage

After the quenching step, the sample is injected to a fluidics system maintained at 0 $^{\circ}\text{C}$ in the cold box (Figure 1a; MéCour Temperature Control, LLC, Groveland, MA, USA). The quenched solution is pumped over a pepsin column (104 μL

bed volume) filled with porcine pepsin immobilized onto POROS 20 AL media (Thermo Scientific, San Jose, CA, USA) with 0.05% TFA (200 $\mu\text{L}/\text{min}$) for 2 min (for a formic acid-based quench) or 3 min (for a TCEP-based quench), and proteolytic products are collected by a POROS R2 trap column (4 μL bed volume) (Figure 1b). Subsequently, the peptide fragments are eluted from the trap column and separated by a C18 column (5 μm Hypersil Gold, 1 mm \times 50 mm, Thermo Scientific) with a linear gradient of 13% buffer B to 35% buffer B over 23 min (buffer A, 0.05% TFA in water; buffer B, 95% acetonitrile, 5% buffer A; flow rate 30 $\mu\text{L}/\text{min}$) in elution configuration (Figure 1c). The trap and analytical column are washed with 100% buffer B for 6 min at 30 $\mu\text{L}/\text{min}$ in the elution configuration and then for 5 more min at 60 $\mu\text{L}/\text{min}$ in back-flush configuration (Figure 1e). The column is then re-equilibrated to 13% buffer B for 10 min at 40 $\mu\text{L}/\text{min}$. Mass spectrometric analyses were carried out with an LTQ Orbitrap XL mass spectrometer (Thermo Scientific) with a capillary temperature at 275 $^{\circ}\text{C}$ [49] and resolution of 15,000 [51].

Protocols and Discussion

Optimization of Quench Condition

The first condition that needs to be optimized in the feasibility stage is the quench condition of an analyte protein. This condition has a great impact on digestion results and the rest of the downstream operations. By using a standard array of quench conditions, it is possible to finish a digestion optimization in one set of overnight experiments (with an automated liquid handling system), with the data analysis performed the next morning. Depending on the presence of disulfide bonds, one of two different routes can be employed.

If the protein has no disulfide bonds, the (A) set of quench conditions is applied (Table 1). The five quench conditions listed here were developed experimentally over a decade of constant use. If everything is equal, the first three conditions (A1)–(A3) are preferred among the five conditions. This is because TCEP is more difficult to wash away from the system and those quench buffers with TCEP require a longer digestion and desalting time, which results in a slightly higher back exchange (lower deuterium retention; see [Experimental](#)).

A blank injection should be made after each sample injection. The purpose of the blank injection is to check for the presence of carryover. Carryover makes the analysis of HDX study stage very difficult and may lead to faulty results [52, 53]. If carryover is observed, a method to eliminate it should be found in the feasibility stage, as discussed in the next section.

If the protein has disulfide bonds, the (B) set of quench conditions is applied. Digestion of a heavily disulfide bonded protein is one of the major technical challenges for HDX-MS because of its pH, temperature, and time constraints during the analysis [52, 53]. All conditions in the (B) set are urea-TCEP mixtures with slightly higher pH than the optimal pH to minimize back exchange. A higher pH should increase the concentration of active reducing species by shifting the

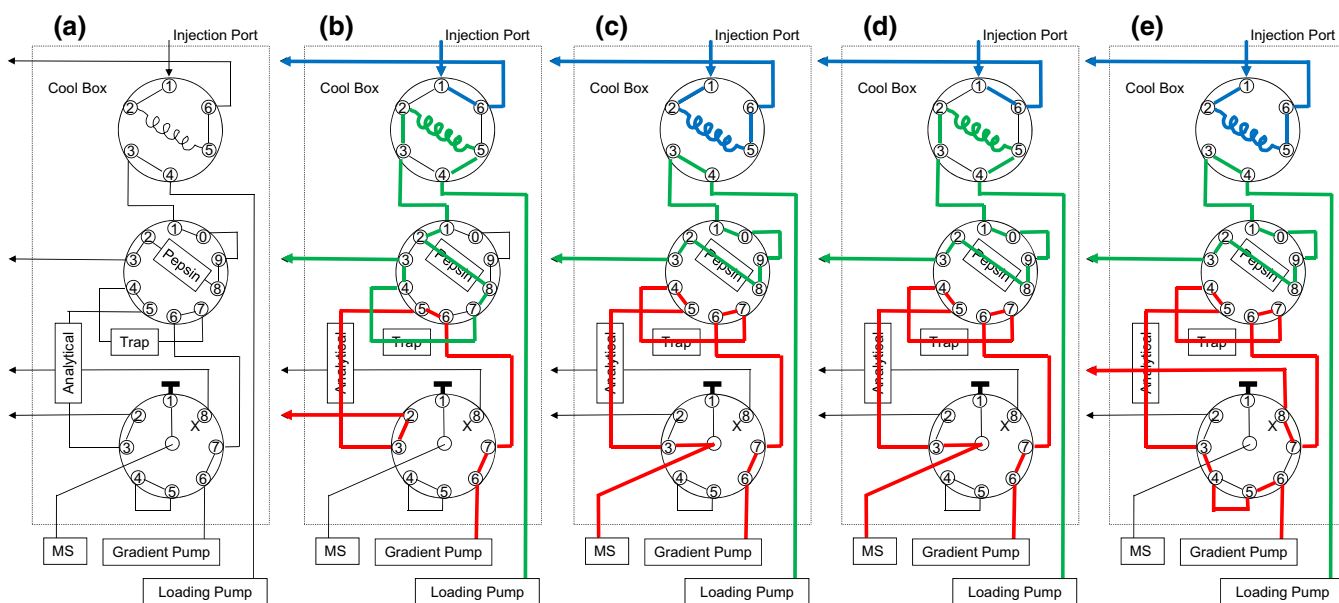
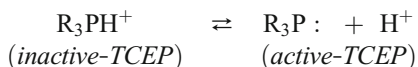


Figure 1. Plumbing of the HDX-MS system. **(a)** All three valves (six-port switching valve, ten-port switching valve, and eight-port selector valve) and three columns (pepsin column, trap column, and analytical column) are in the cold box, which is controlled at 0 °C; **(b)–(e)** show different valve configurations. In each configuration, blue lines are flow path from injection port. Green lines are flow path from loading pump. Red lines are flow path from gradient pump. **(b)** Digestion configuration, in which a loaded protein sample flows over the pepsin column and the digested fragments are collected on the trap column. **(c)** The elution configuration, in which the digested fragments are eluted from trap column, separated by analytical column, and analyzed by MS. In this configuration, washing reagent may be loaded into injection loop. **(d)** Washing reagent injection, in which loaded washing reagent is injected to the pepsin column, while gradient pump is eluting the peptides from the trap column. **(e)** Back-flush configuration, in which the gradient pump back-flushes the analytical column and trap column to waste

equilibrium toward deprotonation to result in better reduction efficiency [54].



On the other hand, the higher pH should in principle induce more back exchange by deviating from the optimum pH. Fortunately, the difference in back exchange we observed was minimal between pH 2.5 and pH 3.0 in urea-TCEP quench buffers. The optimal pH shifts toward higher pH in the presence of high concentration urea, because urea accelerates the acid-catalyzed amide hydrogen exchange reaction and

decelerates the base-catalyzed amide hydrogen reaction [55]. As a result, the average deuterium recovery in our system, when quenched with buffers (B1) or (B2), was around 70%, which is only slightly lower than that with an 8 M urea, 1 M TCEP, pH 2.5 quench. However, deuterium recovery with buffers (B3) or (B4) was down to 50%–55% due to fast base-catalyzed reaction.

The (B5) condition provides useful information in three ways, although the (B5) condition itself is not compatible with the HDX study stage. The quenched solution is incubated at room temperature for a prolonged duration in the (B5) condition and most of the deuterium originally attached to the analyte peptides would be lost. First, the (B5) condition can

Table 1. Standard run list for Quench Condition Optimization

Protein	(A) No disulfide bonds	(B) Disulfide bonds
Target protein	(A1) 1.6 M GuHCl, 0.8% formic acid, pH 2.3	(B1) 2 M urea, 1 M TCEP, pH 3.0
Blank	(A1) 1.6 M GuHCl, 0.8% formic acid, pH 2.3	(B1) 2 M urea, 1 M TCEP, pH 3.0
Target protein	(A2) 3.2 M GuHCl, 0.8% formic acid, pH 2.1	(B2) 8 M urea, 1 M TCEP, pH 3.0
Blank	(A2) 3.2 M GuHCl, 0.8% formic acid, pH 2.1	(B2) 8 M urea, 1 M TCEP, pH 3.0
Target protein	(A3) 3.2 M GuHCl, 0.8% formic acid, pH 2.7	(B3) 2 M urea, 1 M TCEP, pH 4.0
Blank	(A3) 3.2 M GuHCl, 0.8% formic acid, pH 2.7	(B3) 2 M urea, 1 M TCEP, pH 4.0
Target protein	(A4) 2 M urea, 1 M TCEP, pH 1.7	(B4) 8 M urea, 1 M TCEP, pH 4.0
Blank	(A4) 2 M urea, 1 M TCEP, pH 1.7	(B4) 8 M urea, 1 M TCEP, pH 4.0
Target protein	(A5) 8 M urea, 1 M TCEP, pH 2.5	(B5) 8 M urea, 1 M TCEP, pH 4.0*
Blank	(A5) 8 M urea, 1 M TCEP, pH 2.5	(B5) 8 M urea, 1 M TCEP, pH 4.0*

GuHCl: Guanidine hydrochloride, TCEP: tris(2-carboxyethyl)phosphine hydrochloride

*Quench incubation at 23 °C for 3 h

help decide terminating “infeasible” experiments quickly. If an analyte protein *cannot* be digested in this rather harsh condition, either the project should be discontinued or a different protein construct (one which is easier to digest) should be considered. Second, if the (B5) condition gives good digestion of an analyte protein, the peptides identified in (B5) condition should be searched in the other HDX-compatible condition digests. This is because pepsin does not change its cleavage sites depending on the digestion conditions and the (B5) condition usually has higher chance of identifying reduced peptides due to better reduction of disulfide bonds. Third, the disulfide bonded peptides can be searched in the other HDX-compatible condition digests. With fully reduced peptides [from the results of (B5) digestion] and disulfide bond connections (from the collaborator or the literature), it should be possible to calculate the mass of disulfide bonded peptides.

The criteria for the best digestion condition may vary depending on the purpose of a project. The sequence coverage (the number of residues covered by identified peptides with high quality) of an analyte protein is the most important criterion in most projects. The more backbone amide hydrogens that can be monitored, the more information on the protein can be obtained. The second most important criterion is the resolution that can be expected by the peptide subtraction method in the protein [47]; the higher the resolution, the more precise the information that can be obtained. However, there may be regions of special interest in a target protein depending on the purpose of the project (e.g., the complementarity-determining regions of an antibody for paratope mapping). In such a case, the sequence coverage and the resolution of the regions of the interest may be the most critical factors.

Elimination of Carryover

Another technical challenge in HDX-MS is minimizing carryover [56, 57]. During an HDX-MS analysis, some “sticky” peptides may be retained in the system temporarily to be eluted in the next run. When this happens in an HDX study stage, the isotope envelopes of the peptides may become bimodal. In this case, one peptide peak includes more deuterated fraction from the latest injection and less deuterated fraction from the earlier injections. To avoid any confusion in the HDX study stage, minimization of such carryover in the feasibility stage is critical.

Four ways to minimize carryover from the system have been devised; (1) addition of detergent in the quench buffer, (2) extra washing of the protease column by a chaotrope or detergent, (3) back-flushing of the trap column and the analytical column, and (4) usage of PEEK frits or PEEK coated frits in place of stainless steel frits for the columns. It is known that the most common place for peptides to stick is the protease column [57] and the first two relatively easy operations may alleviate many carryover issues.

The first and easiest method to prevent carryover at the protease column is the addition of low concentration Fos-choline-12 to the quench buffer (Fos-choline-14 also works). The detergent reduces the carryover by solubilizing the

peptides, modifying the column surface, or both [58]. Approximately 0.1% of Fos-choline-12 may be added to the quench buffer [CMC of Fos-choline-12 = 1.5 mM (0.053%)]. The detergent does not interfere with the analysis of peptic fragments, because it elutes out at a retention time similar to most intact proteins and a lot later than most peptides (Table 2). Some detergents, such as β -OG or CYFOS-6, elute out with the peptic fragments, suppressing the signal of co-eluting ions, and lowering the signal-to-noise ratios of the peptides. Other detergents, such as DDM and C12E8, were not seen to elute from the analytical column with our standard HDX-MS LC conditions. The long-term effects of these detergents that do not elute out from the system are not clear, although they do not appear to interfere with the analysis of the peptides in the short term.

The second method is extra washing of the protease column, which is performed while the peptic fragments are being separated by analytical column (Figure 1c and d). A chaotrope or detergent solution can be loaded to the system in the Figure 1c configuration and then injected over the protease column in the Figure 1d configuration. The advantage of this method is the freedom to use the chaotropes and detergents that may have detrimental effects on the analysis (e.g., washing with GuHCl when urea is used for quench, or washing with β -OG which interferes with the chromatography). This is because the washing reagent does not go over the protease column with the analyte protein. Multiple washing cycles are possible by toggling the loading (Figure 1c) and injection (Figure 1d) configurations. This procedure can be automated by using an extra vial of chaotrope or detergent in the reagent tray and modification of the liquid handler sequence. Also, this washing protocol does not add any extra time, because one washing cycle (loading and injection) takes about 3 min and the wash is performed during the peptide elution.

The third method requires a selector valve and plumbing configuration compatible with back-flushing of the analytical column and trap column to reduce carryover in the area. In a few cases, carryover may originate in the trap column and analytical column [56]. Back-flushing keeps carryover materials from flowing through the column(s) and is likely to remove them more efficiently than the forward flushing. The plumbing shown in Figure 1 enables back-flushing of the analytical column and trap column by the gradient pump (Figure 1e). Therefore, both columns can be back-flushed by 100% buffer B (95% acetonitrile) in the system. Depending upon the plumbing configuration and valve switching used, this process can be performed as part of the standard experimental process (see [Experimental](#)).

The fourth method is a general consideration to replace stainless steel frits with 0.5 μ m PEEK frits, or PEEK-coated frits, whenever applicable. This replacement should reduce the retention of proteins and peptides that stick to stainless steel surfaces due to electrostatic effects [59]. The replacement has been effective, even for components that were ‘passivated’ stainless steel. In general, stainless steel frits demonstrate increased back pressures from fouling compared with the PEEK frits with the same porosity.

Table 2. Detergents and their Elution Properties

Abbreviation	Chemical name	Nominal mass	Elution
β -OG	n-Octyl- β -D-glucopyranoside	292	with peptides
CYFOS-6	6-Cyclohexyl-1-hexylphosphocholine	349	with peptides
Fos-choline-12	n-Dodecylphosphocholine	351	with proteins
DDM	n-Dodecyl- β -D-maltopyranoside	510	not coming out
C12E8	n-Octaethylene glycol mono-n-decyl ether	538	not coming out

Utilization of Pepsin Specificity Rule

Here we propose to use the pepsin specificity rule for protein quality checking. Our statistical analysis revealed that pepsin does not cleave proteins after arginine, histidine, lysine, proline, or one residue after proline [60]. Some research groups use this rule to eliminate falsely identified peptides before generating a peptide map during the HDX-MS feasibility stage [42, 47]. While this is a valuable exercise, the practitioner may overlook issues in the protein sample that result in confusing data by doing so.

The peptides that appear to violate the pepsin cleavage rule can be indicators for the contamination of truncated proteins. If the protein sample is homogeneous and the pepsin cleavage rule is very strict, there should not be any peptides with the pepsin rule violation. However, if the protein sample contains other proteins, pepsin rule-violating peptides may be observed. Figure 2a shows a pepsin digestion map of a protein sample with nine pepsin rule-violating peptides (shown in red) and all violating peptides have very high Sequest Xcorr scores. In this case, we believe the presence of these violating peptides is due to the contamination of truncated proteins, which is likely the result of proteolytic degradation. For example, pepsin digestion of truncated proteins 1-32 and 33-158 can generate peptides 20-32 and 33-41, respectively. This hypothesis is supported by the fact that all violating peptides disappeared after affinity purification (Figure 2b).

In this case, simple deletion of pepsin rule-violating peptides only hides the presence of degraded fragments in the

sample. For example, with the presence of truncated protein 1-32, peptide 1-13 may be originated partially from the full length protein 1-158 and partially from the truncated protein 1-32. This means that the peptide 1-13 reports the sum of HDX behaviors of the truncated protein 1-32 and the full length protein 1-158. If the truncated protein 1-32 is significantly more dynamic than the full length protein 1-158, artificial bimodal isotope envelopes may be observed for this peptide.

This argument can be expanded to a more general term, that the presence of many pepsin rule-violating peptides may indicate the presence of contaminating proteins. HDX-MS practitioners tend to search a small library of protein sequences [47, 61] and use lower thresholds for peptide search engine scores compared with proteomics. The reasons for these practices are, (1) the goal of this stage is to identify as many peptides as possible to cover the entire sequence of an analyte protein and not to identify at least one peptide for one protein, (2) a protein sample for HDX-MS study is usually assumed to be (relatively) pure, and (3) MS/MS fragmentation may not be very efficient at times. When the sample contains protein impurities, the peptides from the impurities may be falsely assigned as the peptides from the analyte protein, due to the small size of searching library and the low score threshold. In these cases, there is a good chance that some of the falsely assigned peptides violate the pepsin rule.

We suggest not to delete pepsin rule-violating peptides until the generation of a peptide map. This is because the presence of many violating peptides can be used to indicate the presence of contaminating proteins, such as truncated proteins. If there are

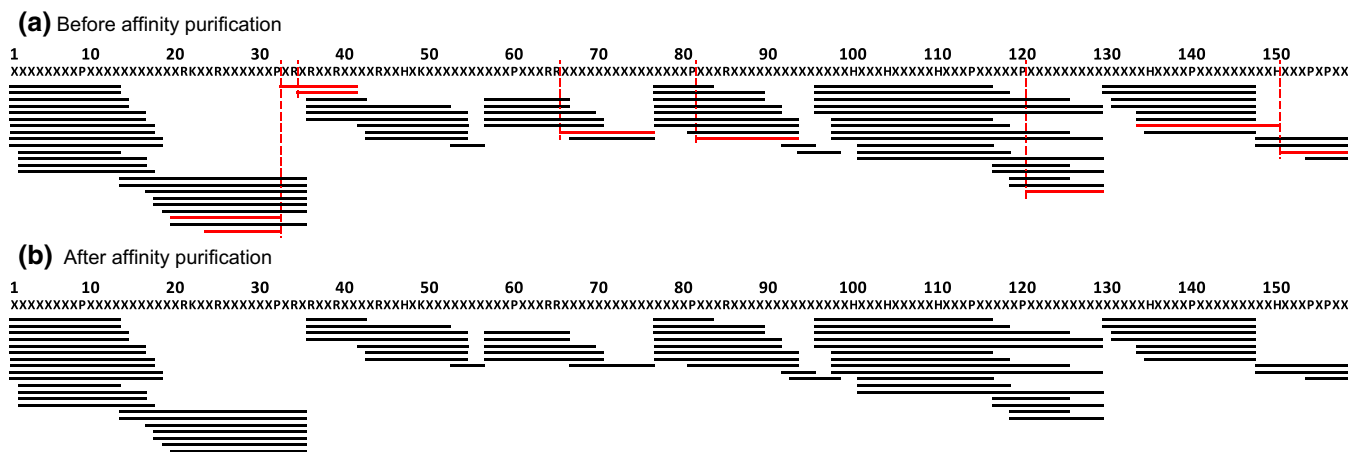


Figure 2. Peptide coverage map of a protein sample; (a) before and (b) after affinity purification. Black lines are identified peptides. Red lines are identified peptides with pepsin rule violation. While there are nine pepsin rule violating peptides before affinity purification (a), no pepsin rule violating peptides are observed after the purification (b)

many pepsin rule-violating peptides in the peptide map, re-purification or re-production of the protein sample should be considered, instead of simply deleting them and proceeding to the HDX study stage.

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