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Sub-Zero Temperature Chromatography for Reduced Back-Exchange and Improved Dynamic Range in Amide Hydrogen Deuterium Exchange Mass Spectrometry

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Abstract

Amide hydrogen/deuterium exchange is a commonly used technique for studying the dynamics of proteins and their interactions with other proteins or ligands. When coupled with liquid chromatography and mass spectrometry, hydrogen/deuterium exchange provides several unique advantages over other structural characterization techniques including very high sensitivity, the ability to analyze proteins in complex environments, and a large mass range. A fundamental limitation of the technique arises from the loss of deuterium label (back-exchange) during the course of the analysis. A method to limit loss of label during the separation stage of the analysis using sub-zero temperature reversed-phase chromatography is presented. The approach is facilitated by the use of buffer modifiers that prevent freezing. We evaluated ethylene glycol, dimethyl formamide, formamide, and methanol for their freezing point suppression capabilities, effects on peptide retention, and their compatibilities with electrospray ionization. Ethylene glycol was used extensively because of its good electrospray ionization compatibility; however formamide has potential to be a superior modifier if detrimental effects on ionization can be overcome. It is demonstrated using suitable buffer modifiers that separations can be performed at temperatures as low as -30°C with negligible loss of deuterium label, even during long chromatographic separations. The reduction in back-exchange is shown to increase the dynamic range of HDX MS in terms of mixture complexity, and the magnitude with which changes in deuteration level can be quantified.

INTRODUCTION

Hydrogen/deuterium exchange (HDX) of proteins has long been an important tool in structural biology for the study of conformational dynamics.¹ The ability to monitor the exchange rates of amides on the protein backbone provides unique insights into the conformational ensemble of the native state as well as interactions with other proteins or ligands. Since Zhang and Smith first coupled HDX to mass spectrometry (MS), mass spectrometry has been increasingly utilized as a detector for hydrogen/deuterium exchange experiments.² The methods greatest advantage is the use of a labeling mechanism that results in minimal structural perturbation. However, this labeling approach suffers from the liability that even under optimal conditions, some loss of label, also referred to as back-exchange (as the label exchanges back into the solvent) typically occurs.³ This loss occurs

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ASSOCIATED CONTENT

Supporting Information. Additional information as noted in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

during all stages of post quench processing and analysis at various rates depending on the exact physical conditions (i.e., low pH digestion, reduction, and subsequent preparation/separation, sequence) and is extremely problematic when site specific information is required from backbone amides with inherently high back-exchange rates.

The dependency of amide HDX rates on pH, temperature, and type of neighboring side chains in unstructured peptides has been well documented.^{2a, 4} In the context of HDX MS, a significant amount of effort has been put into optimizing experimental conditions to control or minimize back-exchange, especially when proteolytic fragments need to be separated.^{2a} Fast chromatographic separations at 0°C and pH 2.5 have been the standard mode of analysis and under these conditions most amides have half-lives ($t_{1/2}$) of 30–120 minutes.⁵ However, the side chains of H, D, C, N and G protect the amide backbone less efficiently or even catalyze the exchange, which results in accelerated exchange. In order to retain the deuterium label, chromatography and other processing has therefore to be limited to just a few minutes with many examples in the literature advocating chromatography to be completed in less than 20 minutes^{2a, b, 5–6}. Restricting the separation to such a short time period can reduce the number of peptides that can be identified and tracked in a mixture, and generally limits the complexity of the samples that can be analyzed, especially when using slower scanning or lower resolution mass spectrometers. In addition, the short time allocated to the separation makes it difficult to implement capillary chromatographic systems which have typically higher elution delays due to larger relative in system dead volumes.

There have been numerous attempts to experimentally limit back-exchange including extremely fast LC⁶, super critical fluid chromatography⁷, and replacement of the HPLC buffer with a non-exchangeable solvent.⁸ However, from the predicted “intrinsic rates” for unstructured peptides based on Bai and Englander’s studies,^{2a, 4} it is apparent that decreasing the temperature from 0°C to –30°C would reduce the rates of back-exchange for backbone amides by a factor of ~40 on average. A reduction of that size would allow ample time to perform extensive chromatographic separation or subsequent sample processing. Importantly, such a level of back-exchange would facilitate the analysis of amides that would normally be lost within a very short period of time at 0°C (i.e., H, D, and C side chains), and could be particularly important for methods that require more extended analysis time, such as “high resolution” HDX MS which employs ETD based peptide fragmentation for deuterium localization. This approach is inherently slower than single stage MS based approaches because of the need to acquire high quality tandem mass spectra for each interrogated peptide.⁹

To exploit the temperature dependence of the exchange mechanism to reduce back-exchange, sub-zero temperature reversed-phase chromatography was implemented. Chromatography at temperatures below the freezing point of water has been used extensively within the fields of cryo-enzymology¹⁰, and biochemistry¹¹ where the reduced temperature is used to slow down enzyme function or reaction kinetics and provide a platform for the study of dynamic systems. To our knowledge, the benefits of sub-zero temperature chromatography have never been demonstrated in the context of HDX MS.

In order to perform LC-MS at temperatures below 0°C, buffer modifiers that can reduce the freezing point of commonly used aqueous solvent systems, are mass spectrometry compatible, and do not preclude the use of liquid chromatography are required. Ethylene glycol, dimethyl formamide, formamide, and methanol are explored here as modifiers because of their well-documented freezing point suppression characteristics. Ethylene glycol in particular, has seen previous use as modifier in low temperature chromatography.^{11a} The impacts of these modifiers on various aspects of a typical HDX MS experiment are

evaluated and the potential benefits and feasibility of using these modifiers to perform sub-zero temperature chromatography with HDX MS are discussed.

EXPERIMENTAL

MATERIALS

Human Fibrinopeptide A, *equine* Cytochrome C, ethylene glycol, formic acid, trifluoroacetic acid (TFA), ethylene glycol(OD)₂, D₂-formic acid, acetonitrile (ACN), formamide, dimethylformamide (DMF), methanol, and M2 anti-FLAG monoclonal antibody were purchased from Sigma Chemical Company (St. Louis, MO). D₂O was obtained from Cambridge Isotopes Laboratories, Inc. (Andover, MA). A tryptic digest of Serum Albumin (*Bovine*) was obtained from Michrom Bioresources, Inc. (Auburn, CA).

METHODS

Standard Preparations—A deuterated fibrinopeptide A stock solution was prepared by dissolution of the lyophilized standard (1mg) in 650μL of D₂O. The stock was heated to 60°C for 2 hours and allowed to sit at room temperature for 1 week. The working solution was prepared by dilution of the stock 10X in 50% ethylene glycol(OD)₂. Stock solutions of BSA digest were prepared by adding 500μL of water or D₂O to the lyophilized digests to produce a 1 pMol/μL solution. Non-deuterated working solutions were prepared by dilution of the stock 2X in loading buffer: 100-X-0.25:X:0.25 %v/v (H₂O:buffer modifier:formic acid), where the modifier percentage was equivalent to the percentage in the running buffer. Deuterated BSA digest working solutions were prepared in the same manner by dilution of the deuterated stock solutions (which had been previously heated to 60°C for 2 hours), however deuterated ethylene glycol-(OD)₂, D₂O, and D₂-formic acid were employed instead of ethylene glycol, H₂O, and formic acid.

Green Fluorescent Protein (GFP) with a C-terminal 3xFlag sequence was expressed using standard methods (see Supporting Information).

Epitope Mapping of 3xFLAG-GFP with M2 Anti-FLAG Antibody—A detailed description of the experimental configurations and settings used for sample loading and performing sub-zero temperature H/D exchange experiments including figures can be found in the Supporting Information under the heading LC-MS setup and operation.

H/D exchange mass spectrometry experiments were performed in similar fashion as described in the literature ^{2a, 9a, 12}. For non-deuterated and deuterated controls, 1.4μL of 3X FLAG-GFP solution (~3.6mg/mL) was diluted with 8.6μL of triethanolamine (TEA) buffer (50mM TEA, pH 7.8, 150mM NaCl) that was prepared in water or D₂O, respectively. Complexes were prepared by mixing 1.4μL of 3X FLAG-GFP solutions with a 5X molar excess of M2 monoclonal antibody (10mg/mL in 50mM TEA buffer, 150mM NaCl) and the appropriate amount of TEA buffer to bring the total volume to 10μL. The solutions were allowed to incubate for 1 hour at room temperature. To initiate the exchange reaction, 30 μL of D₂O buffer (50mM TEA in D₂O, 150mM NaCl, 30% v/v ethylene glycol(OD)₂) was added and allowed to exchange for 5, 30, 180, and 1200 seconds on ice. Samples were quenched to low pH by addition of 30 μL of 2.5% formic acid in H₂O before flash freezing in a dry ice/acetone bath and stored at -80°C until analyzed by LC-MS. For analysis samples were thawed for 30 seconds on ice, and then another 100μL of ice cold quench buffer (2.5% formic acid) was added to effect complete melting. Quickly, 25μL of the mixture was injected and analyzed as described using the trapping setup (Supporting Information, Figure S-1b). The gradients and flow rates of the various buffers are described in Supporting Information, Figure S-2.

Quench Flow Analysis of Fibrinopeptide A—The quench flow analysis was performed using a UPLC (Waters Nano Acquity, Waters Corp., Milford, MA) and an LTQ-Orbitrap hybrid mass spectrometer (Thermo Scientific) with an electrospray ionization (ESI) source (Figure S-1c). The UPLC pump delivered quench buffer 49.75:50:0.25 % v/v (H₂O:ethylene glycol:formic acid) at a flow rate of 49.5 μL/min, which was mixed in a mixing tee (Upchurch Scientific, Oak Harbor, WA) with fully deuterated fibrinopeptide A (100 pMol/μL) delivered at a flow rate of 0.5 μL/min by a syringe pump. Different lengths and diameters of PEEK tubing (volumes used included 10, 50, 250, 500, 1000, 5000 μL) were connected to the outlet of the mixing tee to adjust the off-exchange time. The entire assembly was placed inside a polystyrene foam cooler for cooling to the specified target temperatures using ice/dry ice/ethanol baths. A 50cm, 50 μm transfer line was used to connect the PEEK “back-exchange” tubing to the ESI source.

Data Analysis—The Thermo .RAW files were converted into .ms2 files using an in-house program. Subsequently, tandem MS acquisitions were searched using SEQUEST (Thermo Scientific, Waltham, MA) and search results were automatically filtered using DTASelect 2.0 (Yates Lab, The Scripps Research Institute, La Jolla, CA). Searches were performed against either the BSA or 3X FLAG-GFP sequences. HDExaminer (Sierra Analytics, Modesto, CA) was used to determine the level of deuterium uptake. Results were manually validated or corrected using HDExaminer.

Back-Exchange Predictions—Prediction of amide HDX rates for unstructured peptides was performed using Excel spreadsheets available at <http://hx2.med.upenn.edu/download.html>.⁴ The constants used for each residue are included in the Supporting Information as Table S-1.

RESULTS AND DISCUSSION

Practical Considerations for Sub-Zero Temperature Chromatography—Because of the strong dependence of back exchange on both pH and temperature, chromatography in an HDX MS experiment is typically performed at 0°C and pH 2.5. Analysis at this temperature reduces chromatographic resolution, and increases the mobile phase back pressure compared to room temperature; the latter of which can be managed by most commercially available HPLC pumps. Further reduction of the temperature promotes changes in efficiency and selectivity as well as significantly increased back pressure. To better understand the effects of sub-zero temperatures on a reversed-phase chromatography separation, a model first put forth by Antia and Horvath¹³ which describes the dependence of the height equivalent of a theoretical plate (HETP) on temperature (see Supporting Information for a description of the model and parameters used) was employed.

A plot of theoretical plate height vs. the chromatographic velocity in a 50% ethylene glycol solution (Supporting Information, Figure S-3a and b) shows the optimum velocity shifts to lower values at low temperatures and the HETP shows a sharp minimum around the optimum for the −30°C data. The model suggests that efficient chromatography can be performed at low temperatures by working at lower flow rates than traditionally employed. The measured efficiency of a ProZap 2.1×10mm column at different temperatures using reserpine as the analyte is shown in Supporting Information, Figure S-3c (experimental conditions described in supplemental information, too). While the correlation between the theoretical and experimental data is not particularly high, these data provide insight into the potential of sub-zero temperature chromatography, and are consistent with the modeling results in respect to the shift of the optimum flow rates to lower values at sub-zero temperatures relative to the 0°C curve. Both the model and empirical data suggests that high chromatographic efficiency is possible at sub-zero temperatures; however reduced

chromatographic velocities are needed for optimum efficiencies. In practice, losses in efficiency to gain shorter analysis times and increased throughput might be an acceptable trade-off.

A key parameter of the model is molecular diffusion which is dependent on the viscosity and temperature of the buffer employed. For work at sub-zero temperatures, buffer modifiers are used as freezing point suppressants (see next section) that alter the viscosities of the resulting mixtures. Chromatographic efficiency is reduced under low solute diffusion conditions, and it would be preferable to employ modifiers that provide buffer mixtures of minimal viscosity.¹⁴ Ethylene glycol in particular, has a high viscosity (9.8 centipoises for a 50% ethylene glycol, water mixture at 0°C) that would be expected to reduce chromatographic efficiency significantly.

The effect of temperature on mobile phase viscosity and therefore back pressure is another important practical consideration. The pressure increase is proportional to the viscosity of the mobile phase, which is dependent on the choice of buffer modifier besides the temperature itself. For example, the viscosity of a 50% ethylene glycol solution at -30°C is ~49 centipoises¹⁵ and the viscosity of water at 0°C is 1.79 centipoises, resulting in a ~27 fold pressure increase. Modifiers that are less viscous (i.e., formamide, methanol, DMF) offer an alternative to ethylene glycol if back pressure needs to be kept low. UHPLC can mitigate the higher pressures encountered at sub-zero temperatures for the most part.

Buffer Modifiers for Sub-zero Temperature Chromatography—Ethylene glycol, formamide, DMF, and methanol were explored for their freezing point suppression capabilities, effects on peptide retention, and compatibility with electrospray ionization. All of the modifiers are weaker eluents than acetonitrile in reversed-phase chromatography and therefore, would not be expected to cause excessive decreases in retention. Unfortunately, large molar fractions of these modifiers are required for freezing point suppression to reach temperatures below ~-10°C¹⁶ and at the required concentrations effects on retention are significant. To evaluate the effects of these modifiers, a commercially available BSA tryptic digest (50µL, 0.5pmol/µL) was analyzed keeping the temperature of the chromatographic system at a constant 0°C and varying the percentage of modifier in both the “loading” and “gradient” flows from 0 to 30% v/v. The composition of buffer B (0.25% formic in acetonitrile) was invariant. A 60min linear gradient was delivered at a flow rate of 100µL/min into the ESI source of an LCQ-DECA XP instrument. Tandem MS data were acquired in data dependent manner and searched using SEQUEST as described in the experimental section.

In general, as the percentage of modifier was increased, a decrease in retention (especially for more hydrophilic peptides) and chromatographic resolution (Figure 1 shows data using ethylene glycol as a modifier, other data not shown) was observed. The reduction in retention agreed with expectations based on the elutropic series.¹⁷ Formamide caused the smallest retention time shift while it was maximal for methanol. For example, extracted ion chromatograms of the peptide HLVDEPQNLIK showed 0.8, 8.5, 18.5, and 25.8 min. retention time shifts, and 0, 0.55, 0.65, 1.15 min. peak width increases when 30% v/v solutions of formamide, ethylene glycol, DMF, and methanol were used, respectively. The reduction in resolution followed a different trend as formamide produced the smallest perturbation while methanol and DMF showed the largest increases in peak widths. The increase in peak widths can likely be explained by the detrimental effects of decreased retention and increased buffer viscosity on chromatographic efficiency. Both methanol and DMF caused significant losses in retention for the aforementioned peptide resulting in band broadening at the column head during loading. Use of ethylene glycol as a modifier resulted in better retention than with methanol and DMF but likely hampered resolution because of

its high viscosity and corresponding low solute diffusion rates. Unfortunately, the viscosities of all of the modifier/water mixtures were not available for the conditions used, so the viscosities of the pure modifiers are used as approximate guidelines. Interestingly, formamide has a larger viscosity than methanol and DMF, yet affected peak widths least of all the modifiers. From a purely chromatographic perspective, formamide appeared to offer the best performance with the least amount of perturbation of the separation. However, the impact on the ionization process also has to be considered since the analyte ultimately needs to be converted to detectable ion species in the ESI process.

While ethylene glycol and methanol appeared to have minimal effect on the ionization process, a significant shift in peptide charge-state distributions was observed with DMF and formamide as modifiers. Both formamide and DMF produced large shifts towards lower peptide charge-states resulting in losses in the number of spectra that lead to identifications (Table 1). While the origins of the ion suppression caused by the use of formamide and DMF remain unclear they do correlate with their amide chemical nature. Possibly hydrolysis of the amides under the experimental conditions (in solution and the ionization source) could play a role. Additionally, the low volatility of formamide and the high boiling temperatures of both modifiers could hamper desolvation. Considering both the chromatographic and ionization impacts the results suggest that formamide and ethylene glycol are the most suitable modifiers for sub-zero temperature chromatography in the context of HDX MS. The use of formamide would be preferred if a way to minimize the detrimental ionization effects (e.g., perhaps low flow or split flow) could be found.

Back-Exchange Reduction at Sub-Zero Temperatures—In the current work, predictions of intrinsic exchange rates⁴ serve as a reference to evaluate the impact of sub-zero temperature chromatography on back-exchange. Using the quench flow setup (Supporting Information, Figure S-1c), the deuterium content of fully deuterated fibrinopeptide A was measured as a function of temperature and time in a 50% ethylene glycol, 0.25% formic acid solution.

The rates of amide back exchange ($D \rightarrow H$) for fibrinopeptide A decreased substantially as the temperature was decreased from 0°C to −30°C as seen in the plots of Figure 2. For example, after 100 minutes at −30°C, the deuterium content of fibrinopeptide A was measured at 92%, whereas only 25% was maintained at 0°C. It should be noted that deuterium content >~95% could not be measured likely due to systematic deuterium losses within the emitter and heated ESI interface. In general, the measured deuterium content tracked the theoretical values well despite the different buffer system and detection method used in the original work by Englander and Bai. This shows that loss of label can be almost eliminated in the chromatographic stage of an experiment at sub −20°C temperatures.

Sub-Zero Temperatures Provide More Time for Separation and Data

Acquisition—Traditionally, fast liquid chromatography has been used to limit losses of deuterium due to back exchange, however, these short separation and acquisition times can limit the number of peptides that can be accurately identified and quantified due to isobaric and ionization interferences, and limitations in the resolution, dynamic range, and scan rates of mass spectrometers. The reduction in back-exchange afforded by sub-zero temperatures could provide more time for the separation and acquisition of HDX MS data so as to overcome those limitations.

The impact of sub-zero temperature separations on the extent of back exchange was investigated by analysis of a BSA digest (50μL, 0.5pmol/μL). Both the gradient length (0%B to 75%B over 1.5, 10, and 60 min.) and temperature of the separation (0°C, −20°C, and −30°C) were varied in this experiment. Non-deuterated and fully deuterated BSA samples

were manually loaded into a sample injection loop located within a polystyrene foam cooler (Supporting Information, Figure S-1a). The cooler was packed with ice to cool the injection valve and associated plumbing, and the separation was carried out as described in the experimental section. The temperature of the portable freezer housing the analytical column was varied from 0°C to -30°C. The buffer compositions used to generate the gradients for the analyses were 100-X-0.25: X: 0.25 % v/v (H2O: ethylene glycol:formic acid) for buffer A where X was 0, 30, and 40% respectively. The composition for Buffer B was kept constant at 99.75:0.25 % v/v (acetonitrile:formic acid).

Database searching using SEQUEST of the non-deuterated sample (60 min. gradient) was performed and a list of identified peptides that could be tracked under each of the conditions was compiled. HDExaminer was used to compare the non-deuterated and fully deuterated data files to determine the deuterium incorporation levels for each peptide in the list. Poor quality data was removed by manual validation of the HDExaminer results, and requiring HDExaminer scores of > 0.7 (annotated as “medium to high confidence”). Evaluation of this data showed that separations performed at decreasing temperatures requiring increased levels of ethylene glycol as buffer modifier suffer from lower retention and resolution (Figure 3). These effects are due to the buffer composition as well as the reduced temperatures (see practical considerations for sub-zero chromatography section), and give rise to losses of some hydrophilic peptides as well as a gradual broadening in peak width. However, the loss in chromatographic performance was accompanied by a significant increase in the measured deuterium levels (Table 2). The results clearly show that peptide deuterium incorporation levels were significantly higher when chromatography temperatures below 0°C were employed rather than short gradients at 0°C. In fact, the data shows a 27% increase on average in the percent deuterium incorporation for peptides analyzed using the 60 min. gradient at -30°C compared to the 10 min gradient at 0°C.

Deuterium Retention for Improved Dynamic Range—Due to the reduction in back exchange rates, sub-zero temperature chromatography also provides an avenue for the measurement of rapidly back exchanging amides with improved dynamic range. As demonstration, the protection on the 3X FLAG epitope as a result of binding to the M2 anti-FLAG antibody was measured. The 3X FLAG sequence (DYKDHDGDYKDHDIDYKDDDDK) contains several fast exchanging amides (i.e., amides with neighboring Asp & His side chains) that back-exchange quickly under typical quench conditions. The predicted $t_{1/2}$ for the 3X FLAG peptide at pH 2.5 and 0°C is ~20 min. and many of the amides will have almost completely off exchanged under these conditions after only a few minutes. In contrast, at -20°C and pH 2.5, the predicted $t_{1/2}$ for the 3X FLAG peptide is > 250 min. and most of the amide deuteration can be maintained during the course of a 30 min. chromatographic separation at all positions.

All samples including non-deuterated and fully deuterated controls, as well as deuterated time points for both the 3X FLAG-GFP and the 3X FLAG-GFP / M2 antiFLAG mAB complexes were prepared in duplicate according to the methods described in the experimental section. Sample sets were analyzed at 0°C and -20°C, respectively.

The results from both analyses show significant protection of the 3X FLAG tagged region (Q18GDYKDHDGDYKDHDIDYKDDDDKM42) of the GFP construct by the M2 anti-FLAG monoclonal antibody. The heat map generated by HDExaminer for the -20°C sample set (Supporting Information, Figure S-4) clearly shows that no other regions were significantly protected and that the vast majority of the protein sequence (~95%) was successfully probed. The resolution with which the epitope was localized was reasonably good, as only three amides outside the 3X FLAG sequence showed significant protection. Previous work suggests only the first four residues of the FLAG sequence (DYKD) are

responsible for binding to the M2 antiFLAG mAb¹⁸, however, little variation in the magnitude of protection across the 3X FLAG sequence was observed. This suggests that either the protection encompasses a region significantly larger than the “true” epitope or that other residues in the 3X FLAG sequence contribute to mAb binding (potentially on a time averaged basis). Perhaps proteolysis with alternative proteases (i.e., XIII) could provide more cleavages within the protected region and aid in further resolving residual contributions to the 3X FLAG epitope.

Focusing in on a subset of data obtained from the +5 charge state of the peptide YFQGDYKDHDGDYKDHDIDYKDDDDKMVSKGEE (Figure 4) shows that the measured deuterium levels obtained at –20°C were 2–2.5 fold higher than those obtained at 0°C. Likewise, when sub-zero temperature was employed, the protection of this peptide (Fig. 4b) was significantly larger in magnitude for all but the longest in-exchange time point. Due to the fast exchanging nature of the 3X Flag tagged region, reduced protection at time points longer than 30sec is a result of slowed but still significant deuteration of the complex compared to the GFP alone, which appears saturated at exchange times longer than 30sec. The large difference in protection is clearly attributable to a reduction in back exchange at –20°C that facilitates retention of the deuterium at amides that otherwise rapidly lose the label. By retaining more of the deuterium label, it is reasonable to expect that the dynamic range with which differences in the deuteration level can be measured increases. This should also improve the accuracy of localization based on peptide differences and/or fragment ion differences, in the case of peptide fragmentation approaches.

CONCLUSIONS

It was shown that it is possible to obtain acceptable reversed-phase chromatographic separations at temperatures as low as –30°C using ethylene glycol, dimethyl formamide, formamide, and methanol as buffer modifiers. This allows back-exchange of amide hydrogen in hydrogen deuterium exchange mass spectrometry to be almost completely suppressed during even prolonged peptide separations. Despite the fact that ethylene glycol's effects on peptide retention, chromatographic resolution, and back pressure are inferior to some of the other evaluated modifiers, it is currently preferred due to its good electrospray ionization compatibility. Formamide is expected to be a superior modifier if some of its detrimental effect on ionization could be overcome and this should be investigated further. The use of sub-zero temperature chromatography led to a significant improvement in the amount of deuterium that was retained by peptide amides. This has significant impact on the dynamic range of HDX MS in terms of mixture complexity, the resolution in residue space, and the magnitude with which changes in deuteration level can be quantified. These improved capabilities are expected to support further extension of the technique to new application areas and facilitate more recent developments in the field of peptide fragmentation based HDX MS by ETD to be more readily implemented.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Evaluation of (a) % ethylene glycol in running buffer on reversed-phase chromatography of a BSA tryptic digest at 0°C. Extracted ion chromatograms and peak widths (b) are shown for the +2 charge state of the peptide HLVDEPQNLIK ($m/z = 653.36$).

Figure 2. Percent deuterium as a function of off-exchange time for fibrinopeptide A at (a) 20°C, (b) 0°C, (c) -20°C, and (d) -30°C. The theoretical percent deuterium is shown as a dotted line for reference.

Figure 3.

(a) Reversed-phase chromatography of a BSA tryptic digest at sub-zero temperatures. Extracted ion chromatograms and peak widths (b) are shown for the +2 charge state of the peptide HLVDEPQNLIK ($m/z = 653.36$). The buffer compositions used to generate the gradients for the analyses were 100-X-0.25: X: 0.25 %v/v (H₂O: ethylene glycol:formic acid) for buffer A where X was 0, 30, and 40% for the 0°C, -20°C, and -30°C separations respectively. The composition for Buffer B was kept constant at 99.75:0.25 %v/v (acetonitrile:formic acid).

Figure 4. (a) Deuteration levels and measured protection (b) for each time point of an epitope mapping experiment for the 3X FLAG-GFP / M2 anti-FLAG complex are shown for the +5 charge state of the peptide YFQGDYKDHDGDYKDHDIDYKDDDDKMVSKGEE, which contains the entire 3X FLAG tag.

Table 1

SEQUEST search results for BSA tryptic peptides using different buffer systems.

Modifier	%	Peptides	Spectra	% Seq. Cov.
Ethylene Glycol	0	88	156	64.4
	10	88	162	64.7
	20	89	164	62.1
	30	63	117	59.1
	40	39	95	44.5
DMF	10	36	65	45.0
	20	30	52	41.1
	30	18	37	25.9
	40	15	28	23.6
Formamide	10	31	54	37.9
	20	32	56	37.2
	30	25	48	32.8
	40	24	47	35.4
Methanol	10	58	114	56.0
	20	51	97	51.6
	30	43	78	39.4
	40	50	88	47.6

Table 2

Measured percent deuterium incorporation * and associated retention times (in brackets) for fully deuterated BSA tryptic digest peptides

Sequence	z	0°C			-20°C			-30°C		
		60min	10min	1.5min	60min	10min	1.5min	60min	10min	1.5min
FKDLGEEHFK	2	28 [17.2]	56 [8.6]	53 [8.2]	73 [12.5]	84 [5.9]	86 [10.7]	85 [6.2]		
FKDLGEEHFK	3	37 [17.2]	58 [8.6]	52 [8.2]	74 [12.5]	84 [5.9]	89 [10.7]	84 [6.2]		
DLGEEHFK	2	42 [15.8]	56 [8.4]	56 [8.1]	75 [10.3]	82 [5.7]	82 [8.0]	89 [5.7]		
GLVLAFSQYLOQC*PFDEHVK	2	61 [31.6]	74 [11.9]	81 [9.1]	87 [25.4]	96 [8.3]	94 [24.0]	96 [9.2]		
SQYLQCC*PFDEHVK	2	43 [20.7]	59 [9.3]	64 [8.4]	82 [17.0]	94 [6.4]	87 [15.9]	90 [7.1]		
LVNELTEF	1	53 [25.3]	65 [10.0]	69 [8.8]	78 [22.1]	94 [7.2]	91 [21.0]	92 [8.0]		
LVNELTEFAK	2	67 [23.0]	79 [9.7]	79 [8.6]	81 [19.2]	91 [6.8]	91 [18.2]	91 [7.5]		
SLHTLFGDELCK	2	43 [24.6]	63 [10.0]	69 [8.7]	81 [21.2]	91 [7.1]	90 [20.1]	90 [7.8]		
PNTLC*DEFK	2	60 [21.0]	74 [9.3]	72 [8.4]	82 [17.3]	90 [6.4]	92 [16.2]	86 [7.2]		
PNTLC*DEFKADEK	3	65 [21.0]	76 [9.3]	76 [8.4]	67 [17.3]	91 [6.4]	87 [16.2]	83 [7.2]		
AEFVEVTK	2	66 [16.4]	72 [8.5]	78 [8.1]	89 [11.3]	89 [5.8]	88 [9.3]	89 [5.9]		
DAIPENLPPLTADFADKDVCK	2	55 [24.5]	73 [9.9]	78 [8.7]	87 [21.1]	94 [7.0]	96 [20.0]	94 [7.8]		
DAFLGSFLY	1	48 [30.5]	77 [11.5]	75 [9.1]	85 [25.1]	92 [8.1]	86 [23.7]	88 [9.0]		
DAFLGSFLY	1	61 [32.8]	76 [12.3]	79 [9.2]	90 [25.9]	94 [8.5]	89 [24.4]	90 [9.5]		
DAFLGSFLYYSR	2	61 [29.5]	81 [11.2]	81 [9.0]	89 [24.7]	98 [7.9]	94 [23.4]	98 [8.8]		
RHPEYAVSVLLR	2	71 [20.8]	79 [9.3]	83 [8.4]	89 [17.1]	90 [6.4]	94 [16.0]	96 [7.1]		
RHPEYAVSVLLR	3	68 [20.8]	82 [9.3]	82 [8.4]	94 [17.1]	94 [6.4]	95 [16.0]	96 [7.1]		
RPC*FSALTPDETYPVK	2	54 [21.8]	71 [9.5]	76 [8.5]	83 [18.1]	90 [6.6]	92 [17.0]	95 [7.3]		
LFTFHADIC*TLDPTEK	2	48 [26.5]	67 [10.2]	71 [8.9]	89 [23.5]	95 [7.4]	90 [22.4]	95 [8.2]		
KQTALVELLK	2	78 [21.6]	79 [9.5]	81 [8.5]	84 [17.9]	90 [6.6]	89 [16.8]	90 [7.3]		
AVERAGE		55	71	73	83	91	90	91		

* Deuterium content was normalized against the number of residues minus 1 minus the number of prolines.