Published in final edited form as:

Anal Chem. 2010 December 15; 82(24): 10135–10142. doi:10.1021/ac1019792.

ALKYLATING TRYPTIC PEPTIDES TO ENHANCE ESI-MS ANALYSIS

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Abstract

A major limitation of mass spectrometry-based proteomics is inefficient and differential ionization during electrospray ionization (ESI). This leads to problems such as increased limits-of-detection and incomplete sequence coverage of proteins. Incomplete sequence coverage is especially problematic for analyses that require the detection and identification of specific peptides from a protein, such as the analysis of post-translational modifications. We describe here the development and use of aldehyde-based chemistry for the alkylation of peptide primary amines to increase peptide hydrophobicity, providing increased ionization efficiency and concomitant signal enhancement. When employed to modify the peptide products of protein tryptic digests, increased sequence coverage is obtained from combined modified and unmodified digests. To evaluate the utility of alkylation of peptides for selected reaction monitoring (SRM) assays, we alkylated a peptide from the protein Oct 4, known to play a role in regulating stem cell differentiation. Increased chromatographic retention and ionization efficiency is observed for the alkylated Oct 4 peptide compared to its unmodified form.

INTRODUCTION

Hydrophilic peptides and peptides with hydrophilic post-translational modifications (PTMs), such as glycans and phosphates, are biologically important, but they present several analytical challenges for liquid chromatography-mass spectrometry (LC-MS). These peptides are less likely to be retained during reversed-phase chromatography (RPLC) prior to mass spectrometry analysis. In addition, hydrophilic peptides do not ionize as well as hydrophobic peptides during electrospray ionization (ESI), resulting in reduced signal and higher limits of detection.^{1–13} These challenges can be especially problematic when performing selected reaction monitoring (SRM) mass spectrometry assays. It is often desirable to choose peptides with hydrophilic PTMs for quantification so that the various post-translationally modified forms of the protein can be quantified rather than just the total amount of the protein. The choice of a peptide for SRM analysis normally takes into consideration important characteristics such as sequence, length, and physicochemical properties such as hydrophobicity. Unfortunately, when quantification of a particular PTM is desired, trying to optimize the characteristics of a peptide containing it becomes difficult.^{14,15} If a peptide with the PTM of interest is hydrophilic, the lack of chromatographic retention and poor ionization of the peptide can make quantification nearly impossible.

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SUPPLEMENTARY INFORMATION AVAILABLE

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

We show here that this problem can be addressed by alkylating peptides to increase their hydrophobicity. Hydrophobic modification of peptides has been utilized in LC-MS to increase peptide ionization efficiency during the ESI process.^{1–4,16,17} This strategy is based upon the observation that the ESI response of an analyte is related to its surface activity.^{1,2,5–8,18,19} Analytes that are more hydrophobic have a greater affinity than less hydrophobic analytes for the surface of the droplets produced by ESI.^{1,2,5–8,18,19} This high surface affinity of hydrophobic molecules is caused by the decrease in surface free energy obtained by minimizing interactions between the hydrophobic molecules and water, and increasing water-water interactions.¹ As the excess charge that is produced in the ESI process also resides on the droplet surface, those molecules present on the surface are more likely to acquire those charges. The molecules at the surface that compete successfully for excess charge will be detected by mass spectrometry, whereas hydrophilic molecules in the bulk aqueous interior of the droplet are more likely to become components of neutral salts, which will not be detected.^{1,2,5–8,19}

A few different methods have been described for modifying peptides with hydrophobic moieties.^{1–4,9} Two groups have described the labeling of the amine moieties of peptides with N-hydroxysuccinimide esters.^{2,3} In the case of Mirzaei and Regnier, the modification contained both a hydrophobic component and a quaternary ammonium moiety, which imparts the peptide with a fixed charge. The modification of cysteine-containing peptides with various hydrophobic iodoacetamide derivatives has also been reported.^{1,4} This modification strategy has been demonstrated to lower the limit of detection of synthetic peptide standards for use with isotope dilution mass spectrometry.^{16,17}

Here we report the alkylation of peptides using medium-chain-length alkyl aldehydes to react with the peptide primary amine groups, the N-termini and the side chains of lysine, as shown in Figure 1. The process is a reductive alkylation reaction that proceeds through formation of a Schiff base when the aldehyde reacts with the amine, followed by reduction with a borane-pyridine complex. The reaction occurs twice at each primary amine to yield the dialkylated amine. The dimethylation of amines on peptides and proteins using formaldehyde and a reducing agent has been widely reported in the literature.^{20–30} The alkylation of proteins with longer chain aldehydes^{20–22} and of peptides with dialdehydes²⁴ have also been reported. The ability to modify primary amines makes this reaction applicable to all peptides containing a free N-terminus or lysine residue. Alkylating the amino groups increases the hydrophobicity of the peptides leading to increased chromatographic retention and enhanced ionization.

EXPERIMENTAL SECTION

Reagents

Bovine serum albumin (BSA), horse heart cytochrome c, β -lactoglobulin A from bovine milk, the peptide Gly-Gly-Tyr-Arg (GGYR), DL-dithiothreitol (DTT), iodoacetamide (IAA), urea, trifluoroacetic acid (TFA), and ammonium bicarbonate were purchased from Sigma (St. Louis, MO). Hexanal, octanal, borane-pyridine complex (pyridine-BH₃), and 4-methylmorpholine were purchased from Aldrich (Milwaukee, WI) and butanal was purchased from Fluka (Buchs, Switzerland). HPLC grade water and methanol were purchased from J.T. Baker (Phillipsburg, NJ), HPLC grade acetonitrile (ACN) was purchased from Burdick and Jackson (Muskegon, MI), and Suprapur[®] grade formic acid was purchased from EM Science (Gibbstown, NJ). Modified trypsin was purchased from Promega (Madison, WI). The peptides Asp-Ser-Asp-His-Gly-Ala-Arg (DSDHGAR) and Thr-Leu-Leu-Glu-Leu-Ala-Arg (TLLELAR) were synthesized at the Biotechnology Center, University of Wisconsin-Madison.

Trypsin Digestion

The protein samples were reduced with DTT, alkylated with IAA, and then digested overnight using modified trypsin at 37°C. The digested samples were purified using a C18 solid phase extraction column (200mg/4mL, Ultra-Clean™ SPE C18, Grace, Deerfield, IL). Details of the trypsin digestion and subsequent purification can be found in Supplementary Information.

Alkylation of Peptide Amino Groups

An alkylation reaction mixture was made with 40 mM aldehyde, 60 mM pyridine-BH₃, and 50 mM 4-methylmorpholine (to ensure a mildly basic pH) in 3:1 methanol: water. The individual peptides or protein digests were then dissolved in the alkylation mixture at a concentration of 2 µg peptide/µL alkylation mixture, with the exception of the Oct4 peptide used for SRM which was alkylated at 2 ng peptide/µL alkylation mixture. The alkylation reaction was then allowed to proceed overnight at room temperature before evaporating the mixtures to dryness in a vacuum concentrator.

Calculation of Peptide Hydrophobicity Indices

Peptide hydrophobicity indices were calculated using the Sequence Specific Retention Calculator, SSRCalc,^{31–34} which is available online at: <http://hs2.proteome.ca/SSRCalc/SSRCalc.html>. A larger value for the hydrophobicity index from this calculator indicates the peptide is more hydrophobic than one with a lower value.

Direct Infusion Mass Spectrometry

Peptide samples were dissolved in a solution of 0.2% formic acid in 1:1 methanol: water. The alkylated peptide samples were then mixed with their corresponding unmodified peptide so that the final concentration of each peptide was 1µM. The samples were injected at a rate of 4µL/min into a micrOTOF time-of-flight mass spectrometer (Bruker Daltonics, Billerica, MA). Positive ion mode ESI was performed with a potential of 4800 V, with N₂ used as a nebulizer gas at 0.3 bar and as a drying gas at 3.0 L/min, 200°C.

RPLC-MS/MS of Tryptic Digests

The unmodified and butylated tryptic digest samples were dissolved in 0.1% formic acid to a concentration of 0.133 picomoles/µL. The hexylated tryptic digest samples were dissolved in a solution of 10% acetonitrile, 0.09% formic acid to a concentration of 0.133 picomoles/µL. On-line reversed phase separation was achieved using a capillary column packed in-house with 10 cm of C18 resin (Magic, 100 Å, 5 µm, Michrom Bioresources, Inc., Auburn, CA). Details of the gradient separation are given in Supplementary Information. Tandem mass spectrometry was performed on an LCQ Deca XP Plus (ThermoFisher Scientific, Waltham, MA). After elution, the ions were electrosprayed at 2.0 kV. Each full mass scan was performed between 100–2000 m/z. This was followed by a zoom scan of the most intense ion from the full scan, then MS/MS scans of the two most intense ions from the full scan using 45% normalized collision energy for fragmentation. The search included variable modifications for methionine oxidation (+16 Da) and carbamidomethylation of cysteine (+57 Da). Depending upon the sample being analyzed, N-terminal and lysine modifications for dibutylation (+112.13 Da) or dihexylation (+168.19 Da) were included as variable modifications. A second variable modification on lysine was included to search for incomplete modification (monobutylation +56.06 Da, monohexylation +84.09 Da). Additional details about the mass spectrometry and search parameters can be found in Supplementary Information.

SRM Analysis of Oct4 Peptide

The Oct4 LysC peptide RTSIENRVRGNLENLFLQCPK containing a C-terminal heavy arginine was synthesized by Thermo Fisher (Ulm, Germany) to 95% $\pm$ 5% purity as assessed via amino acid analysis. For determination of the predominant charge state, LC retention time, and ionization efficiency, this LysC peptide was analyzed using three setups: direct infusion on a Triversa Nanomate (Advion, Ithaca, NY) coupled to an LTQ-Orbitrap XL (ThermoFisher Scientific, Waltham, MA); direct injection on a nanoAquity UPLC (Waters, Milford, MA) also coupled to the LTQ-Orbitrap XL; and direct injection on a nanoAquity UPLC coupled to a TSQ Discovery Max triple quadrupole (ThermoFisher Scientific, Waltham, MA) monitoring all possible y-ion transitions. Reversed phase separation was achieved using C18AQ resin (Magic, Michrom Bioresources, Inc., Auburn, CA). For the nanoLC runs, an initial trapping step was performed for 10 minutes at 1 μ L/min flow at the initial gradient conditions. Then, the separation occurred during a 30 minute linear gradient at 0.2 μ L/min, starting at 100% mobile phase A (0.2% v/v formic acid in water) and ending with 40% mobile phase B (0.2% v/v formic acid in acetonitrile), followed by an increased concentration of acetonitrile (85% mobile phase B) and 20 minute column equilibration. The nanospray emitter voltage used was 2.2 kV for all tune methods.

To generate the tryptic peptide, TSIENR, the solution of the LysC peptide standard (dissolved in 95:5 water/acetonitrile) was digested overnight by direct addition of modified trypsin to a 20:1 peptide-to-enzyme ratio. The resulting digest, containing TSIENR, was analyzed on a TSQ Discovery Max triple quadrupole in SRM mode, monitoring the following transitions: 360.4 $\rightarrow$ 418.4 m/z, 360.4 $\rightarrow$ 531.6 m/z, and 360.4 $\rightarrow$ 618.7 m/z. The gas pressure was held at 1.1 mTorr, Q2 collision energy was 18V, and transition scan widths were 0.5 Da while dwell times were kept at 100 ms. Since TSIENR was not retained, the trapping times were varied or excluded from the method.

RESULTS AND DISCUSSION

Relative Change in Electrospray Ionization Response

Three test peptides GGYR, DSDHGAR, and TLLELAR, were modified with different alkylating reagents to assess the efficacy of the alkylation reaction and the effect of alkylation on ESI response. The three peptides were each modified, in separate reactions, using butanal, hexanal, or octanal. Each modified peptide was mixed in a 1:1 ratio with its corresponding unmodified peptide. The ratios of absolute ion abundances were compared for the modified and unmodified forms of the peptides to determine the change in ESI response due to the hydrophobic modification. Figure 2 shows the relative signal change for each modified peptide form with respect to the signal of the unmodified peptide. Since the mixtures are directly infused into the mass spectrometer, we can conclude that the signal enhancement is due to the modification of the peptides as opposed to an effect of different solvent composition due to changes in chromatographic behavior. The largest change observed was a 34-fold signal enhancement due to hexyl modification of GGYR. Interestingly, for GGYR and DSDHGAR, the largest signal enhancement occurred with the addition of hexyl modifications, rather than the even more hydrophobic octyl modifications. Furthermore, for TLLELAR, a much more hydrophobic peptide initially than the other peptides analyzed, the signal actually decreased with increasing hydrophobicity compared to the unmodified peptide. The SSRCalc hydrophobicity indices for the unmodified forms of the peptides were 3.81 for GGYR, -3.93 for DSDHGAR, and 27.08 for TLLELAR. The data suggest that there is a hydrophobicity range in which peptides produce a maximum ESI signal. Peptides with hydrophobicities that are outside this range, because they are either too hydrophilic or too hydrophobic, do not ionize as efficiently. Based on our data, the addition of hexyl modifications to GGYR and DSDHGAR changes the hydrophobicity of these

peptides so that the hydrophobicity is within the range necessary to produce a maximum ESI signal. On the other hand, increasing the hydrophobicity of TLLELAR moves the hydrophobicity of this peptide outside this range. This phenomenon has been observed previously and it has been suggested that there is a correlation between the nonpolar surface area of a peptide and its ESI response.^{16,17}

Modification of Tryptic Digests of Proteins

The tryptic digests of three proteins, cytochrome c, β -lactoglobulin A, and bovine serum albumin (BSA) were modified with butanal and hexanal. Octanal was not chosen as an alkylating reagent for the tryptic digests because the results of the previous section suggest that even for very hydrophilic peptides, the addition of octyl modifications pushes the hydrophobicity of peptides outside the range needed to maximize ESI signal. The wide range of initial hydrophobicities of the peptides of a tryptic digest led us to hypothesize that the addition of butyl or hexyl modifications would work best to bring the hydrophobicities of as many peptides as possible into the optimal range for ESI. The tryptic digests were then analyzed by RPLC-MS/MS. Figure 3 shows chromatograms of the unmodified, butylated, and hexylated digests of BSA. The hydrophobic modifications dramatically increase the retention time of the peptides, with hexyl modifications causing larger increases in retention time than butyl modifications. Table 1 shows the changes in retention time due to hydrophobic modification of the seven peptides of BSA that were found in all forms: unmodified, butylated, and hexylated. The addition of butyl modifications increased the retention time from the unmodified form of the peptides by 11 minutes on average. The average increase in retention time due to hexylation compared to the unmodified form of the peptides was 22 minutes. As the retention time of the peptides increases, the concentration of acetonitrile in which they elute also increases (13–30% ACN for unmodified, 16–37% ACN for butylated, and 29–46% ACN for hexylated).

Database searching of the tryptic digests showed that different sets of peptides were detected for the modified forms of the digests compared to the unmodified form. Table 2 compares the sequence coverage of the unmodified and butylated samples of the tryptic digests for the three protein standards, and a similar table for the hexylated samples is given in Supplementary Information. In most cases, the modified samples yield somewhat lower sequence coverage than the unmodified ones, with the exception of the hexylated form of β -lactoglobulin A, which shows greater sequence coverage than the unmodified form. Although the sequence coverage is often lower for the modified tryptic digests than the unmodified digests, the combined sequence coverage is greater in all cases except one (hexylated cytochrome c). This increase for the combined sequence coverage results from differences in the sets of peptides observed for the modified samples versus the unmodified samples. For example, by analyzing both the unmodified cytochrome c digest and the butylated cytochrome c digest, the combined sequence coverage is 63%, compared to 48% for the unmodified cytochrome c or 39% for the butylated cytochrome c alone. This increase in sequence coverage obtained by analyzing both hydrophobically modified and unmodified digests could prove useful for the detection of additional PTMs, especially PTMs on initially hydrophilic peptides which may not be observed otherwise.

We hypothesized that the differences in the sets of peptides observed from the modified samples compared to the unmodified ones are due to a combination of changes in ionization efficiency and in reversed-phase chromatographic retention. For hydrophilic peptides that are weakly retained and/or poorly ionized in their unmodified forms, increasing the hydrophobicity through alkylation improves retention and ionization. On the other hand, further increasing the hydrophobicity of peptides that are already quite hydrophobic may cause the peptides to bind irreversibly to the chromatography column stationary phase and to therefore fail to be released during the gradient elution. Based on this hypothesis, we

reasoned that the observed difference in sequence coverage arises from the fact that peptides that are initially hydrophilic prior to alkylation would likely only be detected in the butylated form, while peptides that are very hydrophobic even before alkylation would likely only be detected in the unmodified form. To evaluate this hypothesis we used the sequence-specific retention calculator, SSRCalc^{31–34}, to calculate the relative hydrophobicities of the peptides detected from the butylated and unmodified tryptic digests of cytochrome c, β -lactoglobulin A, and BSA. Figure 4 shows a plot of all the detected peptides arranged according to increasing relative hydrophobicity. These values are all calculated for the *unmodified* form of the peptide. The peptide data points in the plot are marked according to whether they were detected only in the butylated form or only in the unmodified form. Peptides detected in both forms are marked as both modified and unmodified. Figure 4 also plots two lines showing the average hydrophobicity index values for peptides that were only detected in the butylated form (solid line) or only detected in the unmodified form (dashed line). The relatively low average value of 10 for peptides only found in the butylated form indicates that these peptides are relatively hydrophilic prior to modification. For peptides that were only found in the unmodified form, the average hydrophobicity index value is 25, which signifies that these peptides are fairly hydrophobic before the alkylation reaction. In between these two extremes, peptides that were observed in both the unmodified and butylated forms have an average hydrophobicity index value of 19, indicating intermediate initial hydrophobicity. These data are consistent with our reasoning, as peptides that are initially hydrophilic, with low hydrophobicity indices, tend to be found only in the modified form, while peptides that are initially hydrophobic tend to only be found in the unmodified form. Furthermore, peptides that have an intermediate initial hydrophobicity tend to be found in both modified and unmodified forms.

Another method of exploring these data involves approximating the relative hydrophobicity of the peptides after modification with butanal. This approximation employed SSRCalc, as before, but two leucine residues were added to the peptide sequence of every dibutylated amino acid (e.g. butyl-modified AEFVEVTK is entered into SSRCalc as LLAEFVEVTKLL). It is reasonable that two leucines, each having a 4-carbon alkyl side chain, would have similar hydrophobicity to that of a dibutylated N-terminus or a dibutylated lysine. To validate this approximation, the hydrophobicity indices of the unmodified peptides were plotted against their retention times. The retention times used were obtained from the results of database searching following RPLC-MS/MS analysis. The calculated hydrophobicity indices of the modified forms of the peptides were then plotted against their retention times. If the relationship between hydrophobicity index and retention time is the same for both modified and unmodified peptides, then approximating dibutylation by the addition of two leucines is a valid means to calculate relative hydrophobicity post-modification with butanal. Figure 5 shows the plot of the relationship between retention time and hydrophobicity index for the detected modified and unmodified peptides. The points for the modified peptides cluster together with the points for unmodified peptides. Linear trendlines were calculated separately for the modified and unmodified peptides. The slopes of these two trendlines differed by only 9%, indicating that the relationship between relative hydrophobicity and retention time is nearly the same for the modified peptides as it is for the unmodified peptides. Therefore, the addition of two leucines for every dibutylated amino acid results in a valid approximation of relative hydrophobicity following alkylation with butanal.

In order to better understand the role that peptide hydrophobicity plays in determining which peptides are detected in RPLC-MS/MS, the hydrophobicity indices of all tryptic peptides from cytochrome c, β -lactoglobulin A, and BSA were calculated. The combined list of peptides for these three proteins was generated by performing *in silico* trypsin digestion. For inclusion on the list, the peptides needed to have at least four amino acids, and not contain

any missed cleavages. The peptide hydrophobicity indices were calculated for all of these tryptic peptides in both their unmodified form and after butyl modification (using the “two-leucine” method). The peptides were binned according to their hydrophobicity indices (<10, 10–20, 20–30, etc.), and then the percentage of peptides detected in each bin was calculated by dividing the number of detected peptides in a given bin by the total number of peptides with hydrophobicity indices in that bin. The resulting histogram is shown in Figure 6. Only a small percentage of the possible peptides at the extremes of the hydrophobicity range are detected, for example, none of the peptides having hydrophobicity indices greater than 50 were detected during any of the RPLC-MS/MS analyses we performed. Similarly, only a small fraction of peptides with hydrophobicity indices less than 10 were detected. Peptides with hydrophobicity indices in the middle of the range are most likely to be detected.

Our data suggest that peptides that are either too hydrophilic or too hydrophobic are not detected during RPLC-ESI-MS. When analysis of a certain peptide is desired, or when maximal sequence coverage is crucial, one could employ derivatization reactions to move peptide hydrophobicity into the suitable range for detection. We have just shown that alkylation serves to increase hydrophobicity. An alternative strategy for peptides that are initially too hydrophobic would be to modify them with hydrophilic moieties. This could be accomplished by modifying peptides with tertiary amines, which can take on an additional charge due to protonation, or quaternary amines which impart a fixed positive charge.^{28–30} In both cases, the average charge state increases and the hydrophobicity decreases. This could prevent irreversible binding to the hydrophobic stationary phase and thereby facilitate detection of the peptides.

The tryptic digest data were analyzed to determine the apparent yield of the hydrophobic modification reactions. The apparent yield is defined as the total signal obtained for the fully modified form of a peptide divided by the total signal from all of its forms, including the unmodified, fully modified, and any incompletely modified forms. The apparent yield may differ from the actual yield due to differences in the ionization efficiencies of the unmodified and modified forms of the peptides. Therefore, we can consider the apparent yield to be the upper bound of the actual yield. The data from six peptides were averaged together for each of the different alkylating reagents. The apparent yield of the butanal reaction was found to be $93 \pm 2\%$ and the apparent yield of the hexanal reaction was found to be $86 \pm 10\%$.

Application of Hydrophobic Modification to a Peptide Chosen for Selected Reaction Monitoring

One interesting application for the increased RPLC retention and enhancement of ionization obtained through hydrophobic modification of peptides is selected reaction monitoring (SRM). A tryptic peptide from the protein Oct4 was chosen to demonstrate this application. Oct4 is a transcription factor that plays an important role in regulating human stem cell differentiation. Pluripotent stem cells express high levels of Oct4, but during differentiation, Oct4 levels decrease until undetected.³⁵ An SRM mass spectrometry assay could track Oct4 concentration changes with high precision and sensitivity, and therefore provide valuable information for developmental biologists. For development of an Oct4 SRM assay, the heavy-labeled LysC peptide RTSIENRVRGNLENLFLQCPK* was synthesized to be used for Oct4 quantification, but the initial analysis revealed many challenges. This peptide is extremely hydrophobic, has low ionization efficiency, and was most abundant in the +3 and +4 charge states, thereby limiting SRM transition selections. The tryptic peptide, TSIENR, was also evaluated, but was too hydrophilic, being poorly retained in RPLC and having low ionization efficiency. Although RPLC is much preferred to normal phase hydrophilic interaction chromatography (HILIC) for MS analysis, we even attempted to develop an SRM method for TSIENR using HILIC (details are given in Supporting Information). The

TSIENR peptide was moderately retained in this case, but the peak intensity was so low as to be unusable for a quantitative assay.

Because neither the LysC nor tryptic peptides were suitable for SRM analysis in their unmodified forms, we decided to explore the alkyl modification of the TSIENR peptide. TSIENR was butylated, as described in this paper, and its ESI response was compared to that of its unmodified form. The butylated TSIENR has ~30 times higher signal compared to the unmodified TSIENR peptide, and is much more strongly retained in RPLC (Figure 7). This signal increase may result from higher ionization efficiency due to the increase in peptide hydrophobicity. It may also result from increased ionization due to elution in a higher percentage of acetonitrile (~22% versus 0%), which is a more favorable solvent composition for electrospray ionization. The overall result of alkylating the TSIENR peptide with butanal is a very substantial improvement in signal-to-noise, which makes this peptide now suitable for an SRM assay.

The results of this TSIENR experiment indicate that alkylation of peptides for SRM analysis could be a valuable tool for lowering the limit of detection for quantification and providing increased latitude in the selection of SRM-applicable peptides. Since the alkylation reaction we describe can be applied to any peptide with a free N-terminus or lysine residue, almost any peptide of interest for SRM can be made more hydrophobic, allowing researchers to choose hydrophilic peptides and peptides with hydrophilic PTMs for SRM analysis.

CONCLUSION

We have developed a simple, one-step alkylation reaction with facile cleanup to increase the hydrophobicity of peptides. The alkylation reaction is applicable to all peptides containing a free N-terminus or lysine residue. The increase in hydrophobicity leads to the enhanced ionization and increased chromatographic retention of hydrophilic peptides. When the alkylation reaction is applied to tryptic digests, an increase in sequence coverage is observed when the alkylated digest is analyzed alongside the corresponding unmodified digest. This increase in sequence coverage derives from the difference in the sets of peptides observed in the alkylated and unmodified forms. Sequence coverage is especially important for detecting PTMs, and the enhancement in sequence coverage could prove useful for identifying additional PTMs previously undetected because the peptides were too hydrophilic.

The alkylation of the TSIENR peptide from Oct4 led to increased chromatographic retention and enhanced ionization, demonstrating that alkylation of peptides for SRM assays could lead to better limits of detection for quantification. The ability to improve the chromatographic retention and ionization of hydrophilic peptides gives researchers more freedom when choosing peptides for SRM assays. This could be especially useful when a PTM of interest is located on a hydrophilic peptide that would otherwise not be amenable to SRM analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge Joshua Coon and Justin Brumbaugh for their assistance with the Oct4 experiments. SEK was supported by an NHGRI training grant to the Genomic Sciences Training Program (5T32HG002760). This work was supported by the National Heart Lung and Blood Institute (NHLBI) Proteomics Program N01-HV-28182; NIH grant 1P01GM081629 and NIH/NHGRI grant 1P50HG004952.

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Figure 2. Relative Fold Change in Signal Intensity Due to Alkylation

The ratios of the signals of the modified peptides compared to signals of the unmodified peptides are shown. For both GGYR and DSDHGAR, the largest enhancement in signal occurs with the addition of hexyl modifications. The signal of TLLELAR is enhanced slightly by the addition of butyl modifications, but the addition of hexyl or octyl modifications causes a reduction in signal relative to the signal of unmodified TLLELAR.

Figure 3. Chromatograms of BSA Digests

The chromatograms of the (A) unmodified, (B) butanal modified, and (C) hexanal modified digests of BSA. The elution of peptides occurs at higher retention times as the peptides are modified with increasingly hydrophobic alkyl chains.

Figure 4. Hydrophobicity Index of Peptides Prior to Alkylation

The detected peptides from cytochrome c, β -lactoglobulin A, and BSA are arranged in order of increasing hydrophobicity index. The hydrophobicity index is calculated for the *unmodified* form of the peptides. The marker indicates whether the peptides were found in the butanal modified or unmodified form. An x inside a circle indicates that the peptide was found in both the modified and unmodified forms. Peptides with low initial hydrophobicity indices tend to be found only in the butanal modified form while peptides that have high initial hydrophobicity indices tend to be found only in the unmodified form. The average hydrophobicity index for the peptides only found in the unmodified form is indicated by the dashed line, while the average hydrophobicity index for the peptides only detected in the modified form is indicated by the solid line.

Figure 5. Relationship Between Retention Time and Hydrophobicity Index

The hydrophobicity indices of the detected unmodified and modified peptides are plotted against their respective retention times. The hydrophobicity indices of the modified peptides were calculated by adding two leucines to the peptide sequence for every dibutylated amino acid in the sequence. The slopes of the linear trendlines for the modified and unmodified peptides were 1.31 and 1.43 respectively, a difference of only 9%. Since the relationship between retention time and hydrophobicity index follows the same trend for both the modified and unmodified peptides, this validates the “two-leucine” method of calculating the hydrophobicity indices of the modified peptides.

Figure 6. Percentage of Peptides Detected Across Hydrophobicity Index Range

In silico digests were performed for cytochrome c, β -lactoglobulin A, and BSA to generate a list of all peptides. Hydrophobicity indices for all peptides were calculated for both the unmodified and modified forms (using the “two-leucine” method). The percentage of peptides detected in each bin of the hydrophobicity range was calculated. Only a very small fraction of peptides having hydrophobicity indices at the extremes of the range were detected.

Figure 7. RPLC-MS Comparison of Unmodified and Butylated TSIENR

Panel A shows that the unmodified TSIENR peptide is essentially unretained on the reverse phase column—it elutes in under 5 min. despite flowing 100% mobile phase A (0.2% formic acid in water) during this time. Peptides found in the shaded region are so poorly retained that they are not observed when trapping is performed. The low signal for this unmodified peptide is evident from the low intensity value of 3.5×10^3 and the chemical noise peaks observed throughout the run. Panel B shows that after butylation, the TSIENR peptide is suitably retained, eluting at 26 min. (~22% acetonitrile) after a 10 minute trapping step was performed (trapping not shown in figure). In addition, the signal intensity value is now 150 times greater than the unmodified peptide shown in panel A; the signal increase in terms of peak area is ~30 fold, which is less than the 150 fold due to the broadness of the unmodified peak. The increased chromatographic retention and signal intensity makes the butylated TSIENR peptide suitable for an SRM assay.

Table 1
Alkylation Increases Peptide Retention Times

The RPLC retention times of seven BSA peptides detected in the unmodified, butanal, and hexanal modified forms. Since hexanal is the most hydrophobic of the modifications evaluated, the hexanal modified peptides display the longest retention times.

Peptide	Retention Time (min)		
	Unmod	Butanal	Hexanal
GACLLPK	21.6	34.7	46.9
YICDNQDTISSK	22.0	34.1	45.9
AEFVEVTK	23.1	35.6	47.4
EYEATLEECCA	23.6	32.8	44.0
YLYEIAR	25.9	32.9	40.5
HLVDEPQNLIK	26.2	35.7	46.4
LVNELTEFAK	29.3	39.5	50.7

Table 2
Sequence Coverage for Butylated Tryptic Digests

For all three proteins, an increase in sequence coverage is obtained by analyzing both the unmodified and butanal modified forms of the tryptic digests.

Protein	Sequence Coverage		
	Unmod	Butanal	Hexanal
cytochrome c	48%	39%	63%
β -lactoglobulin A	64%	30%	67%
BSA	53%	37%	60%