Published in final edited form as:

Anal Chem. 2015 June 2; 87(11): 5500–5504. doi:10.1021/acs.analchem.5b00249.

Surfactant-Induced Artifacts during Proteomic Sample Preparation

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Abstract

Bottom-up proteomics is a powerful tool for characterization of protein post-translational modifications (PTMs), where PTMs are identified at the peptide level by mass spectrometry (MS) following protein digestion. However, enzymatic digestion is associated with additional sample processing steps that may potentially introduce artifactual modifications. Here, during an MS study of the PTMs of the regulator of G protein signaling 4, we discovered that the use of ProteaseMAX, an acid labile surfactant commonly used to improve protein solubilization and digestion efficiency, can lead to in vitro modifications on cysteine residues. These hydrophobic modifications resemble S-palmitoylation and hydroxyfarnesylation, thus discouraging the use of ProteaseMAX in studies of lipid modifications of proteins. Furthermore, since they target the cysteine thiol group, the presence of these artifacts will inevitably lead to inaccuracies in quantitative analysis of cysteine modifications.

INTRODUCTION

Bottom-up proteomics is a mass spectrometry (MS)-based methodology for protein identification and quantification.^{1–5} It has also been used for characterization of post-translational modifications (PTMs) despite its limitations in comparison to the top-down approach.⁶ In bottom-up proteomics, MS is often used in conjunction with chromatographic separation to analyze peptides generated by enzymatic digestion of proteins. The success of

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The authors declare no competing financial interests.

Supporting Information

Materials, proposed mechanisms for the formation of PM-induced artifacts, additional figures and schemes as noted. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

a bottom-up proteomics experiment hinges upon attaining high sequence coverages, which requires optimized sample preparation prior to MS analysis. A typical sample preparation procedure involves protein solubilization, disulfide reduction, enzymatic digestion, and sample cleanup. Detergents are often used to solubilize and denature proteins to improve their accessibility to enzymatic digestion, thereby producing more peptide fragments, especially for hydrophobic proteins. However, many detergents interfere with liquid chromatography (LC) separation and MS analysis, and must be removed after digestion. Recently, several acid labile surfactants (ALSs) have been designed for proteomic sample preparation.^{7–9} As its name suggests, an ALS degrades in acidic conditions, and its degradation products can be readily eliminated before subsequent LC-MS analysis. Supporting Scheme S1 illustrates the decomposition pathway of a widely used ALS, sodium 3-((1-(furan-2-yl)undecyloxy)carbonylamino)propane-1-sulfonate, marketed by Promega under the trade name of ProteaseMAX (PM).⁹ The hydrophilic head of PM is connected to its hydrophobic alkyl tail through a labile furanyl carbamate group. Hydrolysis of PM produces a hydrophilic zwitterionic species (3-aminopropane-1-sulfonic acid) and a lipophilic compound (1-(furan-2-yl)undecan-1-ol), both of which can be easily removed, by reversed phase solid phase extraction (RP-SPE) and by centrifugation, respectively. Unlike other ALSs, PM hydrolyzes under weakly basic conditions, *e.g.*, over the course of tryptic digestion (pH 8.0, 37 °C), thus eliminating the need for buffer acidification after digestion. Moreover, the hydrophobic degradation product of PM helps to improve the recovery of peptides by preventing their adsorption to plastic during and after digestion. These advantages over other ALSs would seem to make PM the favored surfactant for LC-MS analysis.

A major confounding factor in MS-based PTM analysis is the introduction of artifacts during sample preparation, especially in bottom-up proteomics, which requires additional sample processing steps associated with proteolysis.^{10–12} However, the presence of chaotropic, reducing, or alkylating reagents, detergents and other chemicals can cause artifactual modifications that complicate the spectral interpretation. For example, the unpolymerized acrylamide in polyacrylamide gels can react with a free sulfhydryl group to form a cysteinyl-*S*-propionamide adduct.^{13–15} Cyanate, which is a degradation product from urea, can react with the amino and sulfhydryl groups to produce *in vitro* carbamylation.^{16–17} Some chemical modifications may be mistaken as *in vivo* PTMs, as highlighted in two recent studies. Thibault and coworkers showed that the common silver-staining procedure could introduce artifactual sulfation on serine, threonine and tyrosine residues, and this may be misinterpreted as *in vivo* sulfation, or as phosphorylation if only low-mass accuracy data are available.¹⁸ Mann and coworkers showed that lysine residues could be covalently modified by two acetamide molecules when iodoacetamide was used as the alkylating reagent.¹⁹ The resultant 114.0429-Da mass shift is the same as that caused by the diglycyl modification from the ubiquitin remnant after trypsin digestion, and this could lead to erroneous reporting of ubiquitination sites.

The work presented here was prompted by our recent study on the lipid modifications of the regulator of G-protein signaling 4 (RGS4) from insect cells. RGS4 is a member of the family of GTPase activating proteins (GAPs) which are responsible for switching off the G

protein signaling pathway. It was previously reported that RGS4 contains three potential *S*-palmitoylation sites at Cys95 and Cys2/Cys12 residues, as determined by a radioactive labeling experiment and site mutation.²⁰ We have recently shown that, with optimized sample preparation, MS can be used for direct detection of *S*-palmitoylation.²¹ The matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) MS analysis of the RGS4 tryptic digest revealed the presence of two types of hydrophobic modifications, which were initially assigned as *S*-palmitoylation and hydroxyfarnesylation (structures shown in Supporting Scheme S2) based on their mass shifts and response to the hydroxylamine (HA) treatment. However, these two modifications were found to be ubiquitously present in all cysteine residues, a characteristic of *in vitro* modifications. The present study aims to understand the origin of these modifications and to evaluate whether they could be problematic for PTM analysis.

EXPERIMENTAL METHODS

Materials are detailed in the Supporting Information section.

His-Tagged RGS4 Sample Preparation

His-tagged RGS4 was overexpressed by infection of Sf9 cells with baculovirus, and purified by Ni-NTA magnetic agarose beads according to the QIAexpressionist protocol.²² A small portion of the purified proteins was separated by SDS-PAGE, digested by trypsin according to the ProteaseMAX in-gel digestion protocol,²³ and analyzed on an ultrafleXtreme™ MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) for protein ID.

In-Solution Proteolytic Digestion of His-Tagged RGS4

In-solution tryptic digestion was performed following the ProteaseMAX in-solution digestion protocol.²³ Briefly, a 50-μg aliquot of purified His-tagged RGS4 protein was precipitated with 4 volumes of cold acetone. The protein pellet was solubilized by adding 20 μL of ABC buffer (50 mM, pH 8.0) containing 0.2% PM, and vortexing for 15~20 min. Another 71 μL of ABC buffer was then added to the resultant protein solution. Reductive alkylation was performed by addition of 1 μL of DTT (500 mM) and incubation at 56 °C for 20 min, followed by addition of 3 μL of IAM (500 mM) and incubation at room temperature for 15 min in the dark. Digestion was performed by addition of 1 μL of PM (1%) and 4 μL of trypsin (0.5 μg/μL) and incubation at 37 °C for 3 h. For tryptic digestion without reductive alkylation, 50 μg of purified His-tagged RGS4 was buffer exchanged against 100 μL of Tris (50 mM, pH 7.4)/0.05% PM solution through a 10K MWCO centrifuge filter, followed by addition of 2 μg of trypsin and incubation at 37 °C for 3 h. In both cases, the resultant digests were incubated with 0.5% TFA at room temperature for 10 min to hydrolyze PM and to deactivate trypsin. The insoluble PM degradation product was removed by centrifugation at 16,000 x g for 10 min. The digests were aliquoted, dried and stored at -80 °C for later use.

LC-MS/MS Analysis

RGS4 digests with (RGS4-RA) or without reductive alkylation (RGS4-noRA) were desalted by C18 ZipTip. Briefly, samples were loaded onto C18 ZipTip pipette tips in 5% ACN/0.1%

TFA, eluted with 90% ACN/0.1% TFA after 5 washes with 5% ACN/0.1% TFA, dried down, and re-dissolved in 5% ACN/0.1% FA. LC-MS/MS analyses were performed on an LTQ-Orbitrap XL instrument (Thermo Fisher Scientific, San Jose, CA) equipped with a nanoAcquity UPLC (Waters, Milford, MA) and a Triversa Nanomate system (Advion Biosystems, Inc., Ithaca, NY). Mobile phase A consisted of 99:1 water/ACN with 0.1% FA and mobile phase B consisted of 1:99 water/ACN with 0.1% FA. Samples were loaded onto a Waters Symmetry trapping column (C18, 5 μ m, 0.18 mm ID x 20 mm) at a flow rate of 4 μ L/min and washed with 95% A for 4 min. Separation was performed on a Waters BEH130 analytical column (C18, 1.7 μ m, 0.15 mm ID x 100 mm) at a flow rate of 0.5 μ L/min. The gradient was held at 5% B for 3 min, increased to 95% B over 90 min, and kept at 95% B for 9 min. It was then ramped to 98% B in 1 min, kept at 98% B for 4 min, and ramped down to 5% B over 3 min followed by column re-equilibration at 5% B for 15 min. The MS event cycle consists of one MS scan ($r = 60,000$ at m/z 400) and three data-dependent MS/MS scans ($r = 7,500$), where the three most abundant ions with charge state ≥ 2 were selected with an isolation window of ± 3 m/z for collision-induced dissociation (CID) tandem MS analysis with the normalized collision energy set at 35%. The MS data were processed manually using the Proteome Discoverer software (Thermo Fisher Scientific, San Jose, CA).

MALDI-TOF MS Analysis of the Hydrophobic Peptides

Hydrophobic peptides were enriched using the homemade RP-SPE tips packed with the POROS R1 50 resin. An aliquot of RGS4-noRA was dissolved in 5% ACN/0.1% TFA and loaded onto the POROS R1 50 tip. After 3 washes with 5% ACN/0.1% TFA, the sample was sequentially eluted with 20% ACN/0.1% TFA, 40% ACN/0.1% TFA, and 60% ACN/0.1% TFA. A small portion of the RGS4-noRA digest and each of its three fractions were crystallized with DHB (10 μ g/ μ L in 40% ACN/0.1% TFA) and analyzed on an ultrafleXtreme MALDI-TOF/TOF mass spectrometer. Additional aliquots of the 40% ACN eluent which contained the majority of the hydrophobic peptides were dried down and incubated either in the 50 mM ABC buffer containing 10 mM DTT at 37 °C for 1 h, or in 1 M HA (pH 7.4) at room temperature for 1 h. The resulting peptides were also analyzed on the ultrafleXtreme instrument. The spectra were analyzed with the FlexAnalysis 3.4 software.

RESULTS AND DISCUSSION

Extraction of His-Tagged RGS4 from Sf9 Cells

SDS-PAGE of the purified proteins showed a major band at ~25 kDa (> 90% purity, Supporting Figure S1). This band was excised and subjected to reductive alkylation, in-gel digestion, and MALDI-TOF MS analysis. Peptide mass fingerprinting showed a match of the 25-kDa band to RGS4 with 73% sequence coverage by tryptic digestion, indicating the successful overexpression and purification of RGS4.

Characterization of Hydrophobic Peptides by MALDI-TOF MS Analysis

Although the in-gel tryptic digest of RGS4-RA covered 9 out of 11 cysteine residues, including all three reported *in vivo* palmitoylation sites, no palmitoyl peptide was observed. The absence of palmitoyl peptides could be due to their low abundances and/or facile

palmitoyl loss during sample preparation or MS analysis. It was recently shown that *S*-palmitoylation is unstable in regular ammonium bicarbonate (ABC)-containing tryptic digestion buffer, and that the presence of the reducing agent dithiothreitol (DTT) greatly accelerates depalmitoylation.²¹ Here, following the protocol of our previous study, purified RGS4 was digested in neutral Tris buffer (50 mM, pH 7.4) with PM (0.05%) added to prevent protein aggregation and adsorption of hydrophobic peptides onto plastic surfaces. Since there is no disulfide bond in RGS4, the reductive alkylation step was skipped to minimize potential palmitoyl loss. Enrichment of the hydrophobic peptides was achieved by stepwise elution as described in the Experimental section.

The MALDI-TOF mass spectra of RGS4-noRA and its digestion products (Supporting Figure S2) showed that nearly all peptides were recovered in the 20% and 40% acetonitrile (ACN) elution buffers, with rough separation of the more hydrophilic peptides into the 20% ACN/0.1% trifluoroacetic acid (TFA) eluent and the hydrophobic peptides into the 40% ACN/0.1% TFA eluent. The majority of the hydrophobic peptides contained either modification X (238.19 Da) or modification Y (220.18 Da) (Figure 1a). To further determine the chemical nature of these modifications, the 40% ACN eluent was subjected to either DTT or HA treatment and the MALDI-TOF mass spectra of the DTT- and HA-treated sample are shown in Figure 1b and Supporting Figure S3, respectively. Incubation with DTT or HA resulted in the loss of modification X from all X-modified peptides, whereas modification Y was resistant to DTT and HA treatments. HA cleavage is considered specific to the thioester linkage and has been commonly used to distinguish *S*-acylation from other cysteine modifications. Because modification X resulted in a mass shift close to that caused by palmitoylation (238.230 Da), and was similarly susceptible to DTT and HA treatments, it seemed reasonable to assign it as *S*-palmitoylation. Meanwhile, modification Y was tentatively assigned as hydroxyfarnesylation (220.183 Da) due to their comparable mass shift and similar resistance to DTT and HA treatments.

LC-MS/MS Analysis of Hydrophobic Peptides

To further determine the modification site(s), the tryptic digest of RGS4-noRA was analyzed by LC-MS/MS on an LTQ-Orbitrap instrument. Supporting Figure S4a shows the total ion chromatogram of the 3h tryptic digest of RGS4 analyzed on a nano-C18-UPLC column. Most unmodified peptides were eluted within 35 min, whereas the modified peptides were eluted after 35 min, presumably due to their increased hydrophobicity. Like the MALDI-TOF MS analysis, LC-MS/MS analysis also revealed two types of modifications on these hydrophobic peptides. Figures S4 and 2 show the extracted ion chromatograms and the CID tandem mass spectra of a tryptic peptide and its two modified counterparts from RGS4-noRA. The peptide eluted at 31.3 min ($m/z = 822.8834$, Figure S4b) was identified as FYLDLTNPSSCGAEK ($[M + 2H]^{2+}$, $m/z = 822.8823$) based on its accurate mass and CID spectrum (Figure 2a). This peptide was also detected in the X- and Y-modified forms. The CID spectrum of the X-modified peptide FYLDLTNPSSC_XGAEK ($[M + 2H]^{2+}$, $m/z = 941.9805$, R.T. = 48.2 min, Figure S4c) is shown in Figure 2b. Modification X can be localized to the cysteine residue based on the mass difference between the y_4 and y_5 ions ($m = m_{Cys} + 238.196$). Similar to *S*-acylation, modification X appeared to be labile under CID, as evidenced by the presence of a high-abundance $[M - X + 2H]^{2+}$ ion and several $y -$

X ions (labeled as y^* ions) in the CID spectrum. However, despite having the same nominal mass as palmitoylation and sharing similar chemical and physical properties as *S*-acylation, modification X cannot be assigned as *S*-palmitoylation, as such an assignment would have a mass error that significantly exceeds the range acceptable for an Orbitrap measurement. The CID spectrum of the Y-modified peptide FYLDLTNPSSC_YGAEK ($[M + 2H]^{2+}$, $m/z = 932.9744$, R.T. = 48.5 min, Figure S4d) is shown in Figure 2c. The mass difference between the y_4 and y_5 ions as well as that between the b_{10} and b_{11} ions ($m = m_{Cys} + 220.184$) indicates that modification Y also occurred at the cysteine residue. Consistent with the MALDI-TOF MS result, the accurate mass of modification Y also matches that of hydroxyfarnesylation.

Although the MS analysis positively identified two types of cysteine modifications here, neither modification has been reported on RGS4, and modification X does not match any known PTM in the Unimod database. X and Y appeared to be universal modifications, with either or both occurring on all eleven cysteine residues on RGS4 (Supporting Figure S5a). Such non-specificity is a hallmark of *in vitro* modifications. Moreover, the accurate mass of modification X matches that of the hydrophobic degradation product of PM, and the mass difference between modifications X and Y is simply that of a water molecule. We propose that X- and Y-modified peptides were formed via nucleophilic conjugate addition of the cysteine sulfhydryl group to the hydrophobic degradation product of PM (Supporting Schemes S3, S4). To further investigate the origin of these modifications, tryptic digestion was performed in Tris buffer in the presence of a different ALS, RapiGest. MALDI-TOF-MS analysis of the RGS4 digest in RapiGest (Supporting Figure S6) showed no evidence of peptides carrying either modification X or Y, suggesting that these modifications were PM-induced artifacts. Additional examples of PM-induced artifacts are shown in Supporting Figure S7.

Extent of ProteaseMAX-Induced Artifacts in Proteomic Sample Preparation

Given the wide use of ProteaseMAX in proteomics studies, it is necessary to investigate the extent of PM-induced artifacts in proteomic sample preparation. The analysis so far has been focused on the RGS4-noRA sample, where the reductive alkylation step was omitted to minimize potential loss of *S*-palmitoylation. However, reductive alkylation is commonly used in MS-based proteomic analysis, and since the reducing reagent DTT has a profound effect on modification X, an accurate account of the extent of PM-induced artifacts should be obtained following the routine sample preparation protocol. Supporting Figure S5b shows the modification map of RGS4-RA from the LC-MS/MS analysis of its tryptic digest. Even with reductive alkylation, modification X was still detected on 2 cysteine residues and modification Y on 7 cysteine residues. The effect of reductive alkylation can be evaluated by comparing the relative abundance of X- and Y-modified peptides from the RGS4-noRA and RGS4-RA samples. To compensate for the variation in the sample loading amount, electrospray current, and other factors, the native reference peptide method²⁴ was adopted for ion abundance normalization among different samples. Here, the tryptic peptide LGFLLQK from RGS4 was chosen as the internal reference, since it does not contain any missed cleavage or residue that is prone to *in vitro* modifications. The peak area of the extracted ion chromatograms of the reference peptide and peptides of interest in all observed

charge states was measured manually in the Xcalibur Quan browser, and the normalized peptide abundance was calculated as the ratio of the integrated peak area of the peptide of interest to that of the internal reference peptide.

The relative abundances of 9 cysteine-containing peptides with free thiol, X-, Y-, or carbamidomethyl modification in the RGS4-noRA and RGS4-RA samples are summarized in Figure 3. In the RGS4-noRA sample, the extent of X- and Y-modifications varied substantially from peptide to peptide, possibly influenced by the location of the cysteine residue and the pKa value of its thiol group. Not surprisingly, with reductive alkylation, modification X was either undetectable or significantly diminished in abundance due to its vulnerability to DTT treatment. The observation of a small amount of X-modified peptides, VVTC_XR and GLAGLPASC_XLR, could be due to incomplete alkylation that allowed addition of X to the residual free thiol during the subsequent digestion step. Additionally, the hydrophobic X-groups may have been buried inside PM micelles and protected from DTT cleavage. In contrast, in the sample prepared for MALDI-TOF MS analysis (Figure 1), complete removal of modification X by DTT was achieved because PM was eliminated by hydrolysis under acidic condition prior to the DTT treatment.

Unexpectedly, the level of modification Y was also reduced in the RGS4-RA digest despite its apparent resistance to the DTT treatment (Figure 1). This could be due to the competing reaction of DTT with the hydrophobic PM degradation product. Consistent with this hypothesis, co-incubation of a cysteine-containing peptide standard GCLGNAK with PM and DTT for 3 hours produced negligible amounts of modifications X and Y (Supporting Figure S8b). On the other hand, the Y-modified peptide remained an abundant product when DTT or HA was added after it had already been formed during the 3-h incubation with PM (Supporting Figure S8c, d). Similarly, many Y-modified peptides were detected in the RGS4-RA sample, and they were presumably formed during the protein solubilization step before the addition of DTT. A time-course study on these PM-induced modifications revealed that Y-modification was a slow process, with its level gradually increasing over a span of 5 hours, whereas X-modification was much faster, reaching a high level within 10 minutes. The level of X-modification dropped substantially at later time points, possibly due to its reversible nature and competition from the irreversible Y-modification (Supporting Figure S9). It appears that reductive alkylation can largely eliminate X-modification and significantly reduce the level of Y-modifications, provided that there is not much delay between the addition of PM and the addition of DTT. In an actual proteomic experiment, however, the rate of these modifications will likely vary depending on the accessibility of the cysteine residue and its local environment. The RGS4-RA study showed that these artifacts will remain a tangible problem even with a short solubilization period (Figure 3). It is recommended to revise the ProteaseMAX digestion protocol to minimize the impact of these artifacts.

CONCLUSIONS

The present study shows that two types of *in vitro* cysteine modifications (X and Y) can occur during routine proteomic sample preparation involving ProteaseMAX. Modification X has the same nominal mass and similar chemical and physical properties as S-

palmitoylation, including its gas-phase fragmentation behavior under CID, and this could lead to false reporting of *in vivo* palmitoylation, especially when a low-mass accuracy MS instrument is used. Modification Y has the same elemental composition as hydroxyfarnesylation, likely via the same thioether linkage, making it extremely difficult to differentiate these two modifications by chemical reactions or mass spectrometry. Although the level of PM-induced modifications can be substantially reduced by performing reductive alkylation immediately after a short solubilization period, addition of DTT also causes undesirable loss of *in vivo* S-acylation. Thus, one should be cautious with the use of ProteaseMAX in proteomic sample preparation, especially when studying lipid modifications of proteins. In addition, the current study also calls for an investigation on the impact of these PM-induced artifacts on the accuracy of quantitative analysis of various cysteine modifications because they, too, target the cysteine thiol group.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge financial supports from the National Institutes of Health via grants P41 GM104603, S10 RR020946, S10 OD010724, and NIH/NHLBI contract HHSN268201000031C. We thank Prof. David Atkinson and Prof. Haya Herscovitz for granting the access to the cell culture facility and for their valuable suggestions. We are grateful to Prof. Elliott M. Ross at the University of Texas Southwestern Medical Center for providing the RGS4 baculovirus.

References

1. Gygi SP, Corthals GL, Zhang Y, Rochon Y, Aebersold R. Proc Natl Acad Sci U S A. 2000; 97:9390–9395. [PubMed: 10920198]
2. Aebersold R, Goodlett DR. Chem Rev. 2001; 101:269–296. [PubMed: 11712248]
3. Aebersold R, Mann M. Nature. 2003; 422:198–207. [PubMed: 12634793]
4. Bantscheff M, Lemeer S, Savitski MM, Kuster B. Anal Bioanal Chem. 2012; 404:939–965. [PubMed: 22772140]
5. Zhang Y, Fonslow BR, Shan B, Baek M-C, Yates JR III. Chem Rev. 2013; 113:2343–2394. [PubMed: 23438204]
6. Siuti N, Kelleher NL. Nat Methods. 2007; 4:817–821. [PubMed: 17901871]
7. Yu YQ, Gilar M, Lee PJ, Bouvier ESP, Gebler JC. Anal Chem. 2003; 75:6023–6028. [PubMed: 14588046]
8. Chen EI, Cociorva D, Norris JL, Yates JR. J Proteome Res. 2007; 6:2529–2538. [PubMed: 17530876]
9. Saveliev SV, Woodroffe CC, Sabat G, Adams CM, Klaubert D, Wood K, Urh M. Anal Chem. 2012; 85:907–914. [PubMed: 23256507]
10. Karty JA, Ireland MM, Brun YV, Reilly JP. J Chromatogr B. 2002; 782:363–383.
11. Lubec G, Afjehi-Sadat L. Chem Rev. 2007; 107:3568–3584. [PubMed: 17645314]
12. Beck F, Lewandrowski U, Wiltfang M, Feldmann I, Geiger J, Sickmann A, Zahedi RP. Proteomics. 2011; 11:1099–1109. [PubMed: 21298789]
13. Chiari M, Righetti PG, Negri A, Cecilian F, Ronchi S. Electrophoresis. 1992; 13:882–884. [PubMed: 1483433]
14. Sechi S, Chait BT. Anal Chem. 1998; 70:5150–5158. [PubMed: 9868912]
15. Hamdan M, Galvani M, Righetti PG. Mass Spectrom Rev. 2001; 20:121–141. [PubMed: 11746345]

16. Lippincott J, Apostol I. *Anal Biochem.* 1999; 267:57–64. [PubMed: 9918655]
17. McCarthy J, Hopwood F, Oxley D, Laver M, Castagna A, Righetti PG, Williams K, Herbert B. *J Proteome Res.* 2003; 2:239–242. [PubMed: 12814262]
18. Gharib M, Marcantonio M, Lehmann SG, Courcelles M, Meloche S, Verreault A, Thibault P. *Mol Cell Proteomics.* 2009; 8:506–518. [PubMed: 18936056]
19. Nielsen ML, Vermeulen M, Bonaldi T, Cox J, Moroder L, Mann M. *Nat Methods.* 2008; 5:459–460. [PubMed: 18511913]
20. Tu Y, Popov S, Slaughter C, Ross EM. *J Biol Chem.* 1999; 274:38260–38267. [PubMed: 10608901]
21. Ji Y, Leymarie N, Haeussler DJ, Bachschmid MM, Costello CE, Lin C. *Anal Chem.* 2013; 85:11952–11959. [PubMed: 24279456]
22. <http://web.mnstate.edu/provost/QiaExpressionist.pdf>.
23. <https://www.promega.com/~media/files/resources/protocols/technical%20bulletins/101/proteasemax%20surfactant%20trypsin%20enhancer.pdf>.
24. Ruse CI, Willard B, Jin JP, Haas T, Kinter M, Bond M. *Anal Chem.* 2002; 74:1658–1664. [PubMed: 12033257]

Figure 1.

MALDI-TOF mass spectra of the hydrophobic peptides in the 40% ACN eluent (a) before and (b) after 1-h incubation with 10 mM DTT at 37 °C. X-modified peptides are labeled in blue, Y-modified peptides are labeled in red, and unmodified peptides are labeled in black.

Figure 2. The CID spectra of the doubly protonated peptides (a) FYLDLTNPSSCGAEK, (b) FYLDLTNPSSCXGAEK, and (c) FYLDLTNPSSCYGAEK.

Figure 3. The relative abundances of 9 cysteine-containing peptides with free thiol, X-, Y-, or carbamidomethyl modification in the RGS4-noRA and RGS4-RA digests.