Utility of the Enzymatic Labeling Method for Stable Isotope Incorporation into Protein Digests

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Overview

Purpose

- To demonstrate the use of stable isotopes incorporated into protein digests by enzymatic means as a method of protein quantification and characterization

Methods

- Proteins were digested with trypsin in the presence of natural water ($H_2^{16}O$) or isotopically labeled water ($H_2^{18}O$)
- Analysis of peptides by MALDI-TOF MS

Results

- A mass shift of 2 or 4 Da was consistently observed in peptides digested in $H_2^{18}O$ compared to those digested in $H_2^{16}O$
- Disulfide linked binary peptides could be identified by a mass shift of 8 Da
- Relative quantitation of proteins was demonstrated using a model system

Introduction

Incorporation of stable isotopes is often used in proteomics to study differential protein expression when analyzing by Mass Spectrometry (MS). This may be accomplished by a number of techniques including metabolic, enzymatic and chemical labeling. Each method has limitations and advantages and finds applicability for various proteomics systems. Enzymatic labeling involves the use of proteases to incorporate a mass label, ^{18}O, into virtually all generated peptides.[1,2] This is accomplished by performing digestion in the presence of isotopically enriched water. Since protease digestion is routinely used in proteomic methods, this technique offers the advantage of ease of implementation. Because oxygen atoms in a sample do not spontaneously exchange in the absence of the protease, this method is highly specific for stable isotope incorporation into the carboxy terminus of each new peptide. Only the protein C-terminal peptide will not have a stable isotope incorporated. This peptide can then be easily identified in labeled samples by the lack of a mass shift. These advantages make enzymatic labeling a promising tool for relative protein quantitation with stable isotopes.

Depending on the protease utilized for enzymatic labeling, either one or two ^{18}O atoms can be incorporated into peptides. Trypsin, Lys-C and Glu-C are all capable of incorporating two ^{18}O atoms per peptide. This is due to the nature of the interaction between peptide and protease. These enzymes continue to associate with the peptide fragments following digestion, allowing for equilibration of both oxygens of the terminal carboxyl group. Trypsin digestion in ^{18}O water, therefore results in mass spectra with ion doublets spaced by either 2 or 4 Da. This provides a means of differentiation from analogous samples digested in natural water. In addition, covalently linked binary peptides will display an 8 Da shift when digested in ^{18}O water. This allows for the study of protein structure through identification of internal disulfide bonds and the detection of interacting domains in protein complexes using chemical crosslinking methods.

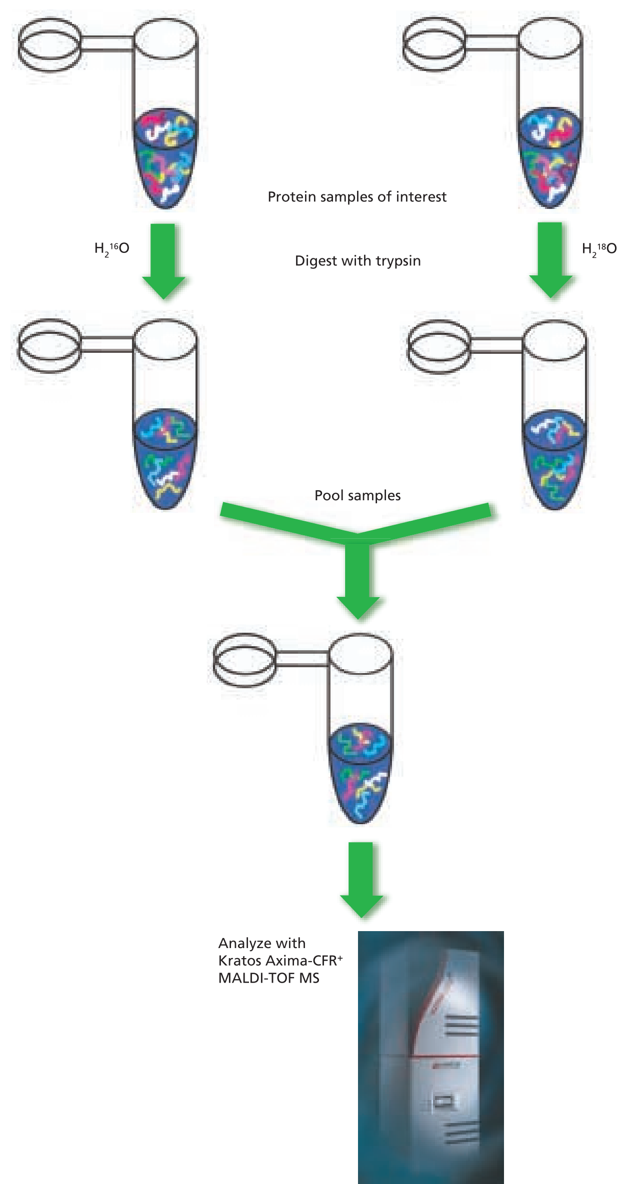
Despite the small mass difference incorporated with enzymatic labeling, relative protein quantitation has been performed with this method. Data confidence can be greatly enhanced by performing inverse labeling.[3] This procedure entails labeling protein populations with both ^{16}O and ^{18}O and then mixing in both combinations to verify that differences in peak intensities are not artificial.

In this study, model proteins or mixtures thereof were tryptically digested in either natural (^{16}O) water or ^{18}O enriched water. The ability to discern disulfide linked peptides as well as to determine relative protein levels was demonstrated.

Materials

- Model proteins: Lysozyme, ß-Lactoglobulin, α-Lactalbumin and Myoglobin (L6876, L0130, L5835 and M0630, Sigma-Aldrich)
- Trypsin, Proteomics Grade (T6567, Sigma-Aldrich)
- Water ^{18}O labeled, 99% isotopically pure (48,709-0, Sigma-Aldrich Isotec)
- ProteoPrep™ Reduction and Alkylation Kit (PROT-RA, Sigma-Aldrich)
- MALDI matrix α-cyano-4-hydroxycinnamic acid (C8982, Sigma-Aldrich)
- ProteoMass™ Peptide MALDI-MS Calibration Kit (MS-CAL2, Sigma-Aldrich)
- All other reagents used were obtained from or prepared at Sigma-Aldrich

Methods



- Prior to trypsin digestion, samples were reduced and alkylated except as noted. Proteins were denatured in 4 M guanidine-HCl, pH 8.5 and reduced with tributylphosphine (TBP) at a final concentration of 5 mM. Alkylation was performed with15 mM Iodoacetamide. Excess reagents were removed by dilution with 50 mM ammonium bicarbonate and ultrafiltration or dialysis.
- Aliquots of approx. 4–20 µg protein in 50 mM ammonium bicarbonate were pipetted into siliconized tubes. Samples were dried in a vacuum centrifuge for at least 45 minutes with no heat.
- Samples were reconstituted in 20 µl of either natural water ($H_2^{16}0$) or isotopically enriched water ($H_2^{18}O$).
- Proteomics grade trypsin was reconstituted similarly.
- Each sample was treated with 0.4 µg trypsin dissolved in the appropriate water. Samples were digested for 12–18 hr at 37 °C.
- Samples were dried in a vacuum centrifuge and reconstituted with 0.1% TFA immediately prior to analysis. Samples digested in each type of water were mixed at a 1:1 volumetric ratio. These were then combined 1:1 with α-cyano-4-hydroxycinnamic acid solution and spotted onto a MALDI target.
- MALDI-MS data was acquired using a Kratos Axima-CFR+ mass spectrometer in positive ion reflectron mode.

Results

Enzymatic Labeling of Peptides

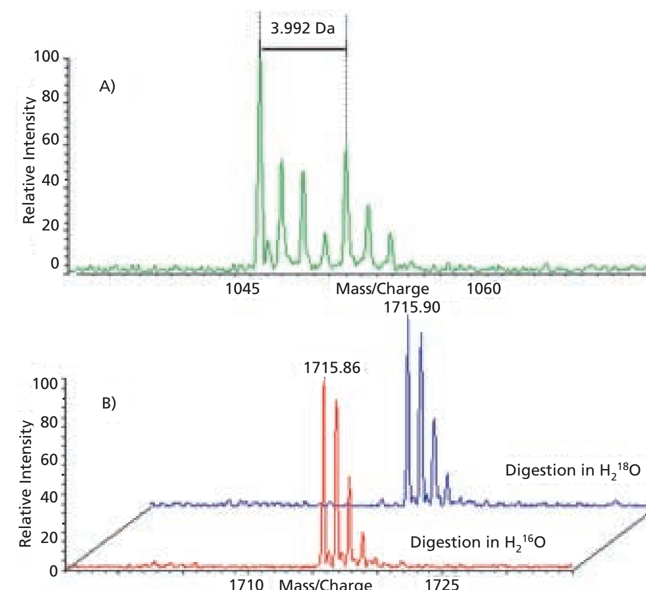


Figure 1: MALDI-TOF MS analysis of tryptic peptide A) GTDVQAWIR ($[M+H]^+$ 1045.87 Da) from lysozyme and B) LSFNPTQLEEQC*HI ($[M+H]^+$ 1715.90 Da) from ß-lactoglobulin. Digestion of proteins was performed in either $H_2^{16}O$ or 99% $H_2^{18}O$. For Spectrum A, the samples were mixed 1:1 and analyzed. This shows the mass shift of 4 Da that is observed for tryptic peptides with the enzymatic labeling method. In Spectrum B, the samples were analyzed separately. This demonstrates the ability to identify the C-terminal peptide by the lack of a mass shift. Alkylated cysteine residues are indicated as C*.

Detection of Binary Linked Peptides

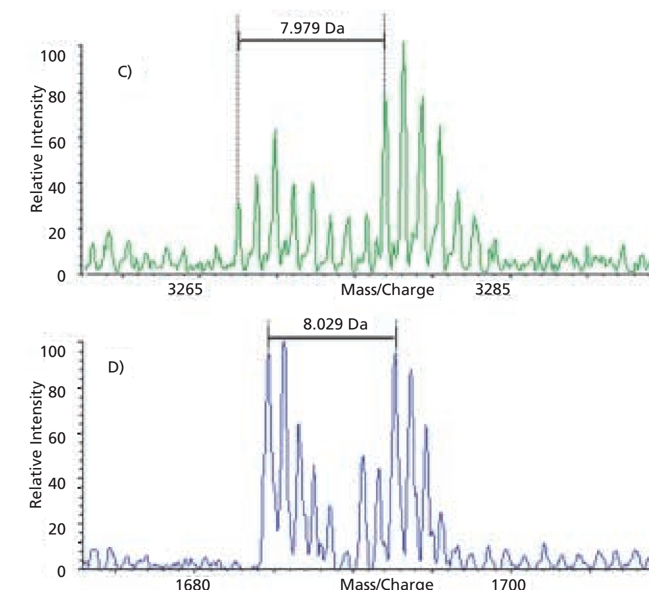


Figure 2: MALDI-TOF MS analysis of tryptic peptides from non reduced and alkylated (C) lysozyme and (D) α-lactalbumin. Spectrum C shows WWCNDGR linked to NLCNIPCSALLSSDITASVNCAK ($[M+H]^+$ 3268.403 Da). Spectrum D shows CEVFR linked to LDQWLCEK ($[M+H]^+$ 1684.639 Da). These spectra represent 1:1 mixtures of samples digested in either $H_2^{16}O$ or 99% $H_2^{18}O$. In both cases, disulfide linked binary peptides were identified by the mass shift of 8 Da.

Quantitation with Enzymatic Labeling Method

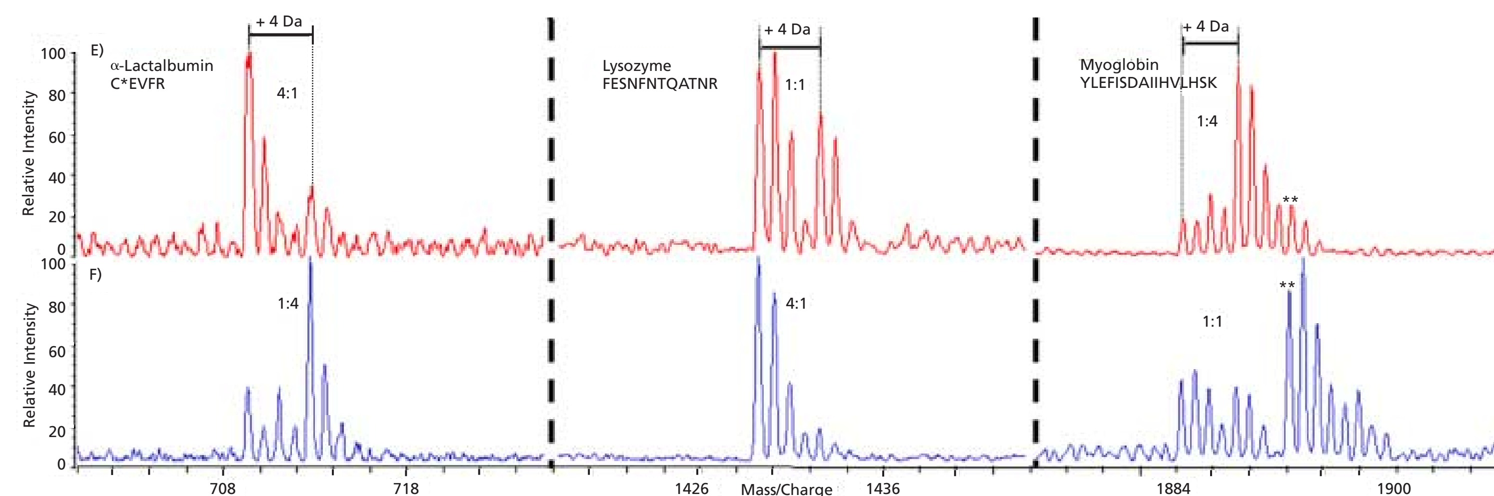


Figure 3: MALDI-TOF MS analysis of a tryptically digested mixture of the proteins lysozyme, α-lactalbumin and myoglobin. The proteins were mixed in various ratios (4:1:1, 1:1:4, or 1:4:1) and digested in the presence of either $H_2^{16}O$ or 99% $H_2^{18}O$ with an inverse labeling method. Each color spectrum (red or blue) illustrates peaks from a single MALDI mass spectrum. Peaks representing each protein are shown. The ratio of protein digested with $H_2^{16}O$ vs. $H_2^{18}O$ is noted. The ** labeled peak is associated with lactalbumin. These spectra demonstrate the potential of the enzymatic labeling method to perform relative protein quantitation. Alkylated cysteine residues are indicated as C*.

Conclusions

- The enzymatic labeling method is a simple technique for specific incorporation of stable isotopes into protein digests.
- Peptides were successfully labeled with ^{18}O using trypsin to incorporate either one or two isotopically labeled atoms.
- Binary linked peptides could be distinguished from non-linked peptides by a shift of 8 Da.
- Differences in relative protein levels were demonstrated using a simple model system and enzymatic labeling.
- The small mass shift of 2 or 4 Da as incorporated with enzymatic labeling is a limitation to the method. Data interpretation can be difficult at higher masses due to the overlap with the natural isotope distribution of carbon but can be clarified with an inverse labeling protocol.

References

1. Yao, X. et al, Proteolytic ^{18}O Labeling for Comparative Proteomics: Model Studies with Two Serotypes of Adenovirus, Anal. Chem., **73**, 2836-2842 (2001).
2. Back, J.W. et al, Identification of Cross-Linked Peptides for Protein Interaction Studies Using Mass Spectrometry and ^{18}O Labeling, Anal. Chem., **74**, 4417-4422 (2002).
3. Wang, Y.K., Ma, Z., Quinn, D.F., and Fu, E.W., Inverse ^{18}O Labeling Mass Spectrometry for the Rapid Identification of Marker/Target Proteins, Anal. Chem., **73**, 3742-3750 (2001).

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