Playing proteomic tag

New peptide-modifying reagents should increase experimental accuracy and reproducibility.

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For proteomic analysis, researchers typically use tryptic digestion of proteins separated by two-dimensional gel electrophoresis (2-DE) and acquire peptide mass spectra using matrix-assisted laser desorption-ionization (MALDI) MS. An alternative, and often complementary, analytical approach involves separating digest peptides by LC followed by electrospray ionization (ESI) MS. In both cases, researchers use software to match peptide masses to corresponding masses calculated from theoretical protein digests in searchable databases. The more complete the constituent peptide identification (degree of sequence coverage), the higher the confidence in any database match. MS/MS peptide fragmentation experiments use similar software to provide information about peptide sequences. Researchers can use this data to reduce ambiguity in identifying a protein when library searches produce multiple matches or in facilitating the de novo sequencing of an unknown protein, such as that from a species not well characterized in the literature.

A difficulty that may be encountered in proteomic investigations, however, is securing sufficient sample to confidently identify proteins of interest. Typical examples include isolating proteins localized in cellular organelles or extracting low-abundant proteins from biological fluids. (The concentration range of proteins in biological fluids such as human plasma spans 9 to 12 orders of magnitude.) Because the effectiveness of protein identification is dependent on the degree of sequence coverage, techniques that improve MS sensitivity can significantly enhance proteomic analysis by detecting a greater number of low-abundance peptides that would otherwise fall below the detection threshold.

Mass-tagging peptides

A technique that has shown promise in enhancing mass spectral detection sensitivity is lysine mass tagging. Proteins undergoing tryptic digestion produce peptides with either a lysine or arginine residue at the C-terminus. In general, the mass spectrometer exhibits lower sensitivity to lysine-terminated peptides than to those that terminate in an arginine residue. This effect is attributed, in part, to arginine's greater basicity and consequently its ability to more readily form a positively charged ion under MS ionizing conditions. This basicity difference has been quantified by comparing the MALDI MS responses for identical peptide pairs differing only in the presence of a C-terminated arginine or lysine residue. In untreated samples, arginine-terminated peptides exhibit a 4–18 times greater signal intensity than peptides terminated with lysine.

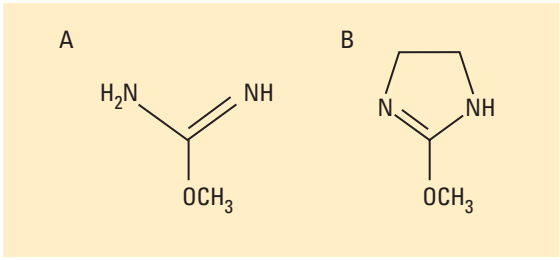

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Figure 1. Playing tag. Researchers can label tryptic peptides by reacting C-terminal lysine residues with *O*-methylisourea (A) and 2-methoxy-4,5-dihydro-1*H*-imidazole (B).

The MS signal of lysine-terminated peptides can be strengthened significantly by converting the lysine ε-amino group to a more basic homoarginine. Researchers have accomplished this transformation by reacting tryptic peptide digests with *O*-methylisourea (OMIU, Figure 1A). The increase in MS signal strength of C-terminal lysine peptides labeled in this way leads to the detection of a greater number of low-abundance peptides and thereby increases sequence coverage in peptide-mapping experiments. For example, OMIU treatment of a tryptic digest of the yeast protein enolase improves detection by MALDI MS such that sequence coverage of the constituent peptides rises from 20% to 46% after derivatization. In addition to the increase in signal strength and contingent sequence coverage, the tagging chemistry provides a more complete estimate of the proportion of lysine-terminated peptides in the digest. The additional information helps to further characterize the complex mixture of digested peptides, improving the efficiency and accuracy of protein sequencing and identification.

Although OMIU labeling improves the MALDI MS signal strength for lysine-terminated digest peptides, the inability to standardize reaction conditions and the tendency to form side products limit the technique's utility. Examination of the literature shows a variety of experimental protocols for OMIU labeling reactions, none of which has been adopted as the standard method. One obstacle in establishing a standard protocol is that reaction times appear to be highly dependent on peptide mass.

Another problem associated with OMIU labeling is its insufficient selectivity, reacting not only with the desired ε-amino groups of lysine but also with the α-amino substituents. There is also evidence of unwanted reaction with N-terminal glycine residues. Although formed in considerably lower yield than the singly labeled ε-amino substituent, the presence of these byproducts can complicate mass spectra. Moreover, byproduct formation is a competing reaction that lowers the potential yield of the desired low-abundance peptide derivatives. The suboptimal increase in signal strength limits the potential enhancement in sequence coverage.

In addition, because OMIU lacks nonlabile positions on which deuterium can be substituted, this reagent is not well suited

for isotope-based quantitation experiments. Thus, the only avenue open for isotopic comparisons requires preparing expensive ^{13}C or ^{15}N derivatives.

Improved lysine tagging

Recently, researchers at the Genomics Institute of the Novartis Foundation developed a new lysine mass-tagging reagent, 2-methoxy-4,5-dihydro-1*H*-imidazole (Lys Tag 4H)—licensed and commercially provided by Agilent Technologies—that overcomes many limitations of mass tagging with OMIU (Figure 1B). MALDI MS experiments demonstrate that the signal from lysine-terminated peptides modified with Lys Tag 4H is 5–25 times stronger than that from the corresponding unlabeled sample. Figure 2 shows the improvement in peptide coverage starting with a typical 2-DE separation. Peak correspondences between original and tagged sample are easily charted because each Lys Tag 4H tag adds a mass of 68 Da.

In addition to the ease with which the Lys Tag 4H labeling protocol can be standardized, there are improvements in reaction selectivity and reductions in byproduct formation compared with OMIU mass tagging. For example, the Lys Tag 4H reaction shows little or no evidence of phosphoserine or phosphothreonine dephosphorylation, peptide deamidation, or other undesired side reactions that have been described in OMIU labeling experiments. Taken together, these enhancements simplify spectral interpretation in both protein mapping studies and the de novo sequencing of unknown proteins, as well as in elucidating structural variations in peptides resulting from nontryptic enzymatic cleavage or from post-translational modification. Lys Tag 4H labeling also simplifies spectral interpretation in MS/MS experiments by generating spectra consisting primarily of y-ions. This result is not obtained with OMIU in a comparable one-step reaction. Figure 3 compares typical MS/MS spectra for a standard peptide labeled with either Lys Tag 4H or OMIU.

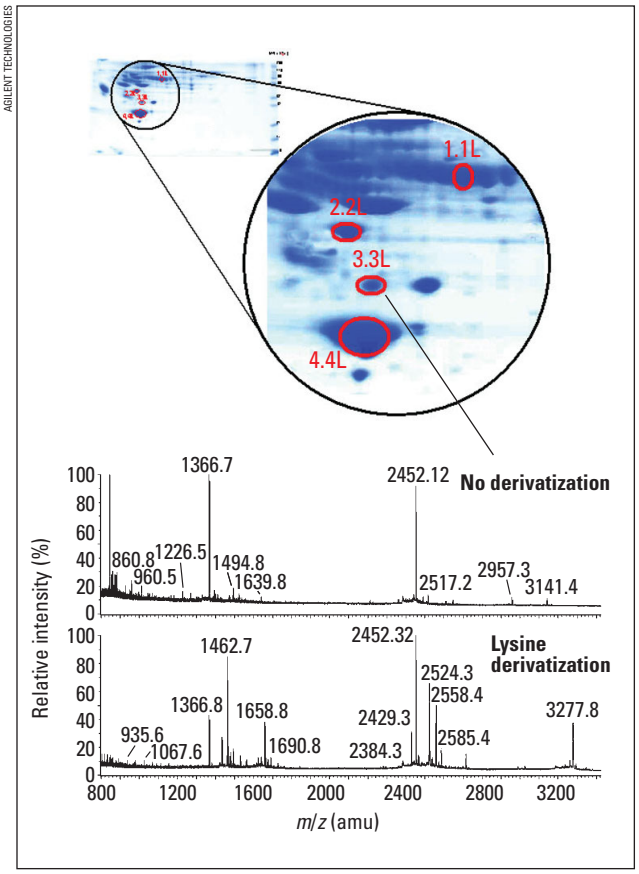

Figure 2. Lysine tagging of in-gel digested peptides. Researchers excised spots from duplicate gels of depleted plasma (top) and performed in-gel tryptic digestion and lysine tagging. They then obtained MALDI MS spectra for the tagged spot (3L) and its untagged control (bottom). They noted that lysine tagging increases signal response of lysine peptides and, therefore, produces better sequence coverage.

Unlike OMIU, which is difficult and expensive to isotopically substitute, Lys Tag 4H is easily prepared as a deuterated compound, thereby enabling quantitative differentiation experiments using isotopically substituted labels. In this technique, researchers digest a control protein sample and an identical sample subjected to some experimental procedure, which they then label with the isotopically substituted (d^4) and the unsubstituted (d^0) reagent (mass difference of 4 Da), respectively. The differentially labeled peptides are then combined and subjected to MS analysis.

By changing the peptide-labeling reagent, researchers have access to a simple cost-effective method for differential quantitation experiments.

The intensity ratio of the corresponding deuterated and undeuterated mass peaks shows whether the experiment altered the concentration of the protein of interest. For example, an intensity ratio of 1:1 indicates no change in protein concentration between the two samples, while an intensity ratio of 5:1 would indicate a fivefold increase relative to the control. Such experiments can be extended, using interval sampling and subsequent differential mass tagging, to chart changing protein concentrations over time. Isotopic labeling can also help researchers characterize peptides by indicating the number of lysine residues present. Because the mass difference between Lys Tag 4H and Lys Tag 4D is 4 Da, dividing the mass separation between corresponding peaks of isotopic analogs by 4 will determine the number of lysine residues in the labeled peptide. This data is especially useful in detecting errant cleavages by trypsin.

Applications

Lysine mass-tagging applications using Lys Tag 4H and Lys Tag 4D are currently being evaluated at a number of beta sites. Gary Siuzdak and his colleagues at The Scripps Research Institute Center for Mass Spectrometry are identifying virus–cell interactions by performing differential quantitation with deuterated Lys Tag 4H to monitor the course of viral infection in a cell.

Similarly, Robert Moritz and colleagues in the Joint Proteomics Laboratory of the Ludwig Institute for Cancer Research at

Royal Melbourne Hospital are using the Lys Tag 4H reagent in conjunction with their development of new protein separation and characterization techniques. Their goal is to identify new proteins that are important in cell growth and in intracellular responses to growth factors—they have a particular interest in developing new proteomic strategies for identifying colorectal cancer biomarkers.

Ole Jensen and his colleagues at the University of Southern Denmark are using Lys Tag 4H in the identification of proteins isolated from microorganisms and diseased tissue; the structural determination of proteins from insect cuticles; the characterization of post-translationally modified proteins; and the investigation of macromolecular complexes formed by protein–ligand, protein–protein, and protein–DNA interaction.

Agilent Technologies is also studying the application of lysine mass tagging in conjunction with a recently developed time-of-flight mass spectrometer. This new device is capable of mass accuracy sufficient for empirical formula determination (±0.02 *m/z*) and a limit of detection, in full scan mode, 10–100 times better than that of conventional linear quadrupole mass spectrometers. This combination of greater accuracy, sensitivity, and speed could accelerate both peptide mapping and de novo protein sequencing.

Techniques that improve MS sensitivity can significantly enhance proteomic analysis by detecting a greater number of low-abundance peptides.

Thus, simply by changing the peptide-labeling reagent, researchers now have access to a simple cost-effective method to synthesize isotopically substituted analogs for use in differential quantitation experiments, and to improve overall peptide detection and therefore sequence coverage.

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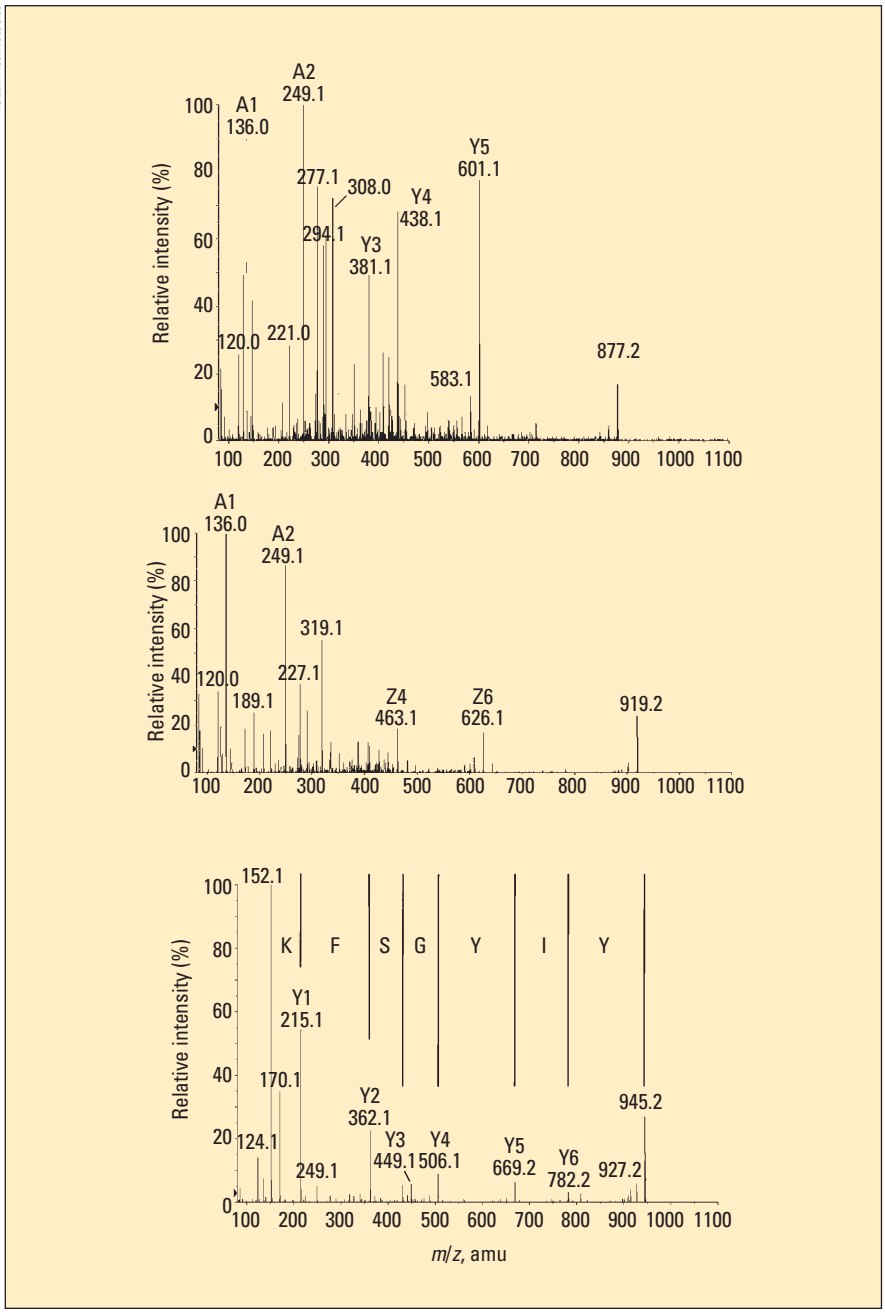

Figure 3. MALDI MS/MS of a standard peptide with the sequence YIYGSFK. Spectrum of the standard peptide (top), the peptide derivatized with *O*-methylisourea (middle), and the peptide derivatized with 2-methoxy-4,5-dihydro-1*H*-imidazole (bottom). The latter derivative yields an uncomplicated series of y-ions that facilitates identification of the peptide sequence.

Further reading

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