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INVITED REVIEW

Analytical strategies for the global quantification of intact proteins

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Abstract The quantification of intact proteins is a relatively recent development in proteomics. In eukaryotic organisms, proteins are present as multiple isoforms as the result of variations in genetic code, alternative splicing, post-translational modification and other processing events. Understanding the identities and biological functions of these isoforms and how their concentrations vary across different states is the central goal of proteomics. To date, the bulk of proteomics research utilizes a "bottom-up" approach, digesting proteins into their more manageable constitutive peptides, but sacrificing information about the specific isoform and combinations of post-translational modifications present on the protein. Very specific strategies for protein quantification such as the enzyme-linked immunosorbent assay and Western blot are commonplace in laboratories and clinics, but impractical for the study of global biological changes. Herein, we describe strategies for the quantification of intact proteins, their distinct advantages, and challenges to their employment. Techniques contained in this review include the more traditional and widely employed methodology of differential gel electrophoresis and more recently developed mass spectrometry-based techniques including metabolic labeling, chemical labeling, and label-free methodologies.

Keywords Proteomics · Intact proteins · Quantitation · Mass spectrometry

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Introduction

Proteomics research is a robust and rapidly evolving research field that seeks to "identify and quantify the structures and biochemical and cellular functions of all proteins in an organism, organ, or organelle, and how they vary in space, time, and physiological state" (Kenyon et al. 2002). While these objectives are yet to be fully realized, many analytical platforms have been developed in pursuit of them. Antibody-based assays such as the enzyme-linked immunosorbent assay (ELISA) and Western blot are frequently employed in laboratories and clinics, often providing sensitive and specific quantification due to the availability of well-characterized antibodies that have been carefully developed to bind to specific protein epitopes corresponding to specific isoforms. However, these assays are often limited in their biological scope, as significant time and cost must be invested in the development of antibody reagents for each protein to be quantified. This renders immune-based techniques impractical for the global quantitative analysis of intact proteins. In this report, we describe strategies that have been developed for the quantitative analysis of intact proteins, including differential gel electrophoresis (DIGE), in which the relative intensity of fluorescent dyed protein spots are quantified within the same gel. We also summarize the direct identification and quantification of intact proteins with mass spectrometry, most notably metabolically labeling strategies in addition to recent developments in chemical labeling and label-free quantification.

Differential gel electrophoresis (DIGE)

Two-dimensional gel electrophoresis (2D-GE) is a widely employed means for protein separation, capable of

visualizing hundreds of proteins and their isoforms separating first by isoelectric point, then by molecular weight. Using traditional visualization protocols such as colloidal Coomassie dye and silver staining, differentially expressed proteins can be compared across replicate gels, although variability across multiple gels negatively affects sensitivity, and these stains lack an adequate linear dynamic range for detection and relative quantification (Quadroni and James 1999; Patton 2000). Succinctly, the poor reproducibility of 2D-GE made it difficult to accurately report real quantitative change in protein expression. As a means of eliminating inter-gel variability, Ünlü and coworkers introduced differential gel electrophoresis, which loads multiple samples that are differentially labeled with fluorescent tags onto the same gel; thus, the proteins are allowed to electrophoretically co-migrate, yet can be detected at different wavelengths and relatively quantified based on fluorescence (Unlu et al. 1997). An overview of the experimental strategy of DIGE is depicted in Fig. 1. In DIGE, fluorescent labeling of proteins is performed prior to 2D-GE in contrast to traditional total gel staining with fluorescent dyes like Sypro Ruby which is performed after separation. Utilizing fluorescent dyes over the usual Coomassie or silver stains provides sensitivity of <1 fmol over a linear range of detection spanning a dynamic range of 10^4 (Viswanathan et al. 2006; Lilley et al. 2002). The most commonly employed reagents for fluorescent labeling in DIGE are synthetic *N*-hydroxysuccinimidyl (NHS) ester derivatives of cyanine dyes including Cy3 and Cy5, which react with primary amine groups (N-terminal α-amino and lysine ε-amino groups) via nucleophilic substitution (Unlu et al. 1997). Minimal labeling is often performed (4–8 pmol of label for each μg of protein), such that <5 % of proteins are labeled. Over-labeling would otherwise result in loss of sample through precipitation. Minimal labeling also reduces interference with downstream mass spectrometric analysis, as a majority of the protein in each spot remains unmodified (Lilley et al. 2002; Gharbi et al. 2002; Tonge et al. 2001). More recently, iodoacetamide and maleimide derivatives of Cy3 and Cy5 have been utilized for labeling cysteines (Shaw et al. 2003). As cysteine residues occur less frequently than lysine, the use of cysteine reactive fluorescent tags allows for greater stoichiometric amounts to be labeled without precipitation resulting in increased sensitivity. Greengauz-Roberts et al. (2005) utilized cysteine-reactive dyes for the relative quantification of 1,000 proteins in microdissected samples of around 5,000 cells, a marked reduction in the amount of sample previously used, and a distinct advantage in the analysis of clinical samples.

While DIGE can potentially allow for the quantification of hundreds of proteins within the same gel, it still presents several challenges. Limitations inherent to 2D-GE are still present in DIGE, including difficulty in characterizing membrane proteins in addition to proteins possessing very high (>150 kDa) and low (<10 kDa) molecular weight, extreme isoelectric points, and low abundance. Differences in molecular weight between fluorescently labeled proteins and their unlabeled counterparts can also result in differential migration of the protein spots and is most apparent at lower molecular weights (Gharbi et al. 2002), causing difficulties for spot picking for MS-based identification, as the unlabeled bulk of the protein will not be present at the fluorescing spot. Often, this issue is addressed with the use of a total protein stain such as colloidal Coomassie or silver stain. Perhaps, one of the greatest pitfalls to DIGE is that although multiple isoforms of a protein may be visualized on the gel, for example multiple combinations of phosphorylation, the specific characteristics of those isoforms are often unobtainable through bottom-up proteomic analysis of the spot. Other variations in DIGE experiments, including fluorescent background (Karp et al. 2004; Karp and Lilley 2005; Kreil et al. 2004), dye biases (Shaw et al. 2003), and spot volumes (Tonge et al. 2001) demand rigorous statistical analyses to establish thresholds for significant quantitative change with an acceptable number of false positives. Even when limits for identifying significant changes in expression have been put into place, attributing changes in fluorescence to a single protein in a spot that may contain several is problematic and requires further validation with more specific quantification methods.

Mass spectrometry-based quantification

Mass spectrometry-based proteomics has led to the development of many platforms that can detect, identify, and quantify up to thousands of proteins from a complex mixture. Two main strategies, termed "bottom-up" and "top-down", are currently employed to carry out proteomics analyses. Bottom-up proteomics, a more widely implemented strategy relative to top-down, involves submitting a protein or protein mixture (e.g., cell lysate) to enzymatic digestion rendering the sample as a complex mixture of short peptides with defined N- and C-termini. The peptides are then separated and analyzed via liquid chromatography (often reversed-phase) coupled to electrospray mass spectrometry (ESI-MS) which measures the intact peptide mass and, subsequently, sequences the peptides using tandem mass spectrometry (MS/MS). Searching the precursor ion mass and sequence information against a predicted protein database often results in hundreds or thousands of protein identifications.

However, sequence coverage of the originating protein structures is often low, with many identifications coming from the detection of a single peptide which may

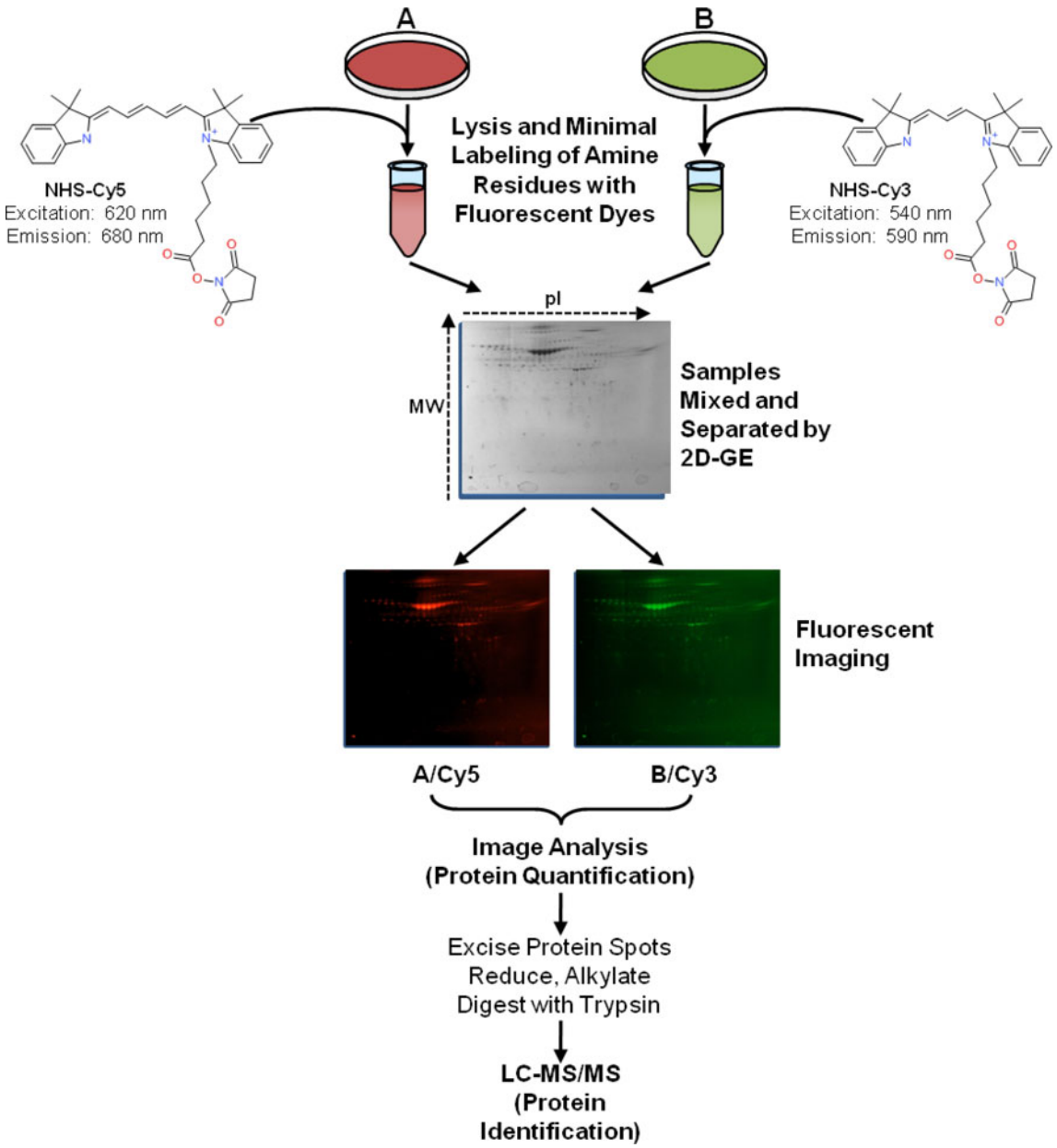

Fig. 1 Overview of differential gel electrophoresis methodology for the relative quantification of proteins resolved on a two-dimensional electrophoretic gel, in which NHS-Cy dyes are reacted with free amine residues in protein samples and co-eluted on a 2D gel. Spots of interest are excised and identified by bottom-up proteomic analysis

correspond to multiple protein forms (Kelleher et al. 1999). These multiple protein forms may each have a unique biological function, potentially possessing diagnostic, prognostic, and/or therapeutic value. Proteins such as brain natriuretic peptide, C-reactive protein, and troponin I and T are already regarded biomarkers in their native forms, but specific modifications (e.g., post-translational modifications (PTMs) and unique cleavages) can result in diagnostic markers that are more specifically related to a disease state (Hawkridge et al. 2005; Das et al. 2003, 2004; Ardelt et al. 1998; Katrukha 1998; Labugger et al. 2000). Knowing the mass of the intact protein, including any modifications that have occurred, which gave rise to a specific proteolytic peptide would be very useful in determining the identity of its specific isoform and whether it is related to a pathological stimulus.

Top-down proteomics provides both the intact mass and sequence information for protein identification and, though not employed as often as the bottom-up technique, continues to mature as a method for identifying and characterizing intact proteins in biological systems (Bogdanov and Smith 2005). The intact protein mass is first obtained

by the mass spectrometer, most often an LTQ-FT-ICR (Syka et al. 2004) or LTQ-Orbitrap mass spectrometer (Hu et al. 2005; Yates et al. 2006). Intact proteins are then isolated for MS/MS analysis by any of a multitude of dissociation techniques [e.g., CID (Jennings 2000), IRMPD (Little et al. 1994), and ECD (Zubarev et al. 1998)] to provide sequence information. The combined intact mass and sequence data enable the specific form of the protein to be determined, locating PTMs and identifying alternative slicing variants and degradation products (Du et al. 2004).

Metabolic labeling for intact protein quantification

Quantitative mass spectrometry most often utilizes the incorporation of stable isotope labels to create an internal standard with similar physical and chemical properties (except for mass) from which reliable quantitative data can be obtained. One of the first attempts at quantifying large, multiply charged, intact proteins by Gordon et al. established that quantification could be achieved using relative abundances of an analyte and a structural analog as an internal standard. However, a three amino acid difference in the amino acid sequence between equine and bovine cytochrome c resulted in hydrophobic differences between the analyte and internal standard. The resulting bias in electrospray ionization efficiency produced two different charge state distributions that limited quantification unless the abundances of all charge states were taken into account (Gordon et al. 1999). This electrospray bias in intact proteins has also been demonstrated in various post-translationally modified isoforms of histone H4, in which methylated and acetylated lysine and arginine residues impart greater hydrophobicity, and thus has greater ionization efficiency over its unmodified form (Pesavento et al. 2006). Even the smallest structural differences have the potential to greatly influence quantification, and so stable isotope labeling has been actively pursued for the quantification of intact proteins.

Metabolic labeling of tissues and cell cultures offer the most efficient means of incorporating stable isotope labels for quantifying intact proteins and have been adapted from protocols for bottom-up analyses to exclude any proteolytic digestion. With metabolic labeling, illustrated in Fig. 2, separate cultures are supplemented with either a light or heavy form of a nutrient [as in $^{15}N/^{14}N$ labeling (Oda et al. 1999)] or amino acid [as in SILAC (Ong et al. 2002)], which are combined 1:1 based on total protein

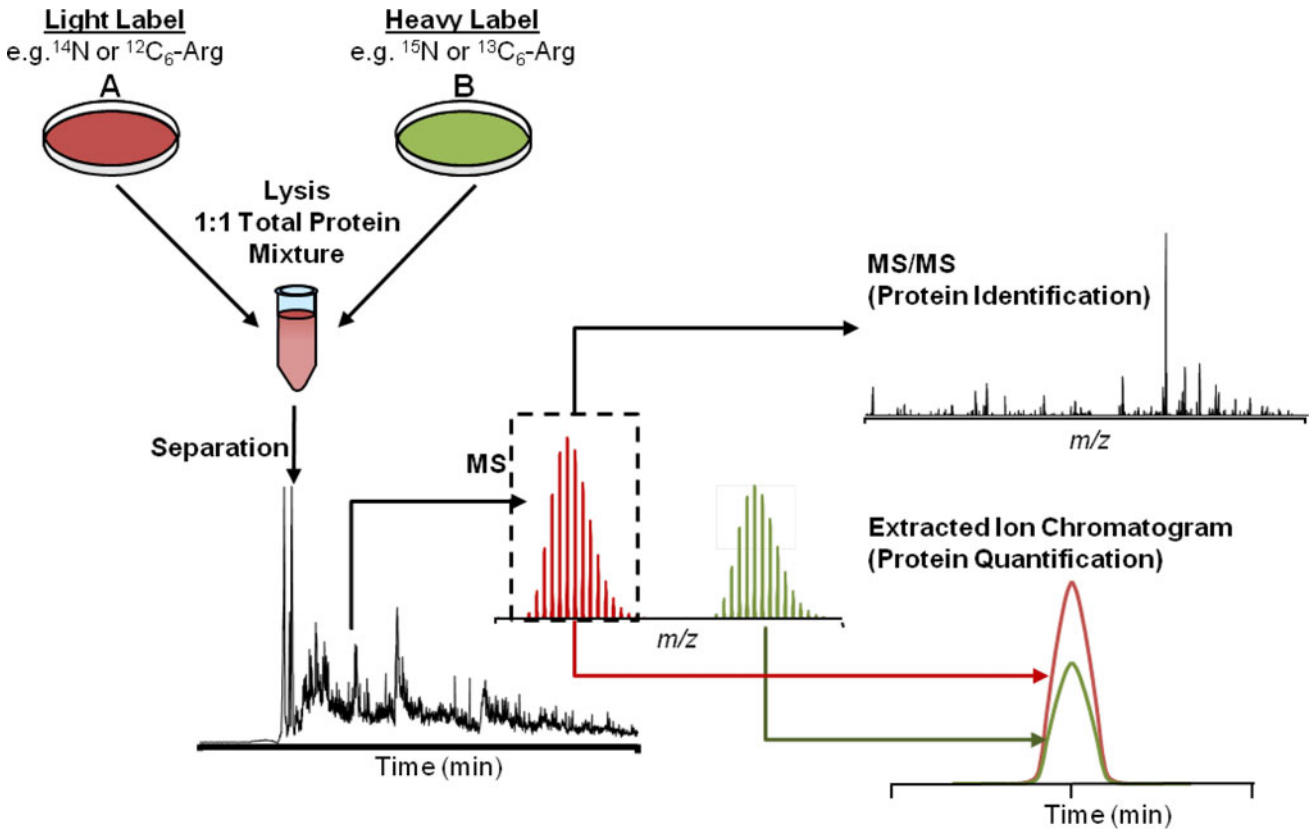

Fig. 2 General outline of a quantitative top-down proteomics strategy employing metabolic labeling with either $^{15}N/^{14}N$ metabolic labeling or SILAC. Labeled proteins are combined 1:1 then separated, detected, identified, and quantified by LC–MS/MS analysis

concentration and carried through a proteomics workflow including sample fractionation, and LC separation and detection using mass spectrometry, followed by bioinformatics analysis where proteins are identified and quantified. Deuterated leucine has been incorporated into model cell cultures, including *E. coli* and *S. cerevisiae*, (Martinovic et al. 2002; Veenstra et al. 2000), which facilitated identification of proteins using accurate intact mass and amino acid counting (Martinovic et al. 2002; Veenstra et al. 2000). However, deuterium labeling slightly reduces the hydrophobicity of labeled species relative to hydrogen. The resulting retention time shifts of up to 30 s in reversed-phase chromatography can hinder quantification, and so deuterated labels are rarely used for top-down quantification (Zhang et al. 2001; Julka and Regnier 2004). Current intact protein quantification strategies employ stable isotopes that do not hinder liquid chromatography retention times. Originally developed by Chait and coworkers for the peptide level analysis of *S. cerevisiae*, $^{15}N/^{14}N$ labeling was first demonstrated for quantitative proteomics by supplementing minimum culture medium with either natural or stable isotope-labeled ammonium sulfate, resulting in total labeling of every nitrogen atom in the protein structure (Oda et al. 1999; Washburn et al. 2002). Kelleher and co-workers used $^{15}N/^{14}N$ metabolic labeling to quantify the yeast proteome and recently employed the technique using top-down LC–MS/MS to obtain 50 protein expression ratios (Parks et al. 2007; Du et al. 2006).

Stable isotope amino acid labeling with $^{13}C_6$-labeled lysine was introduced by Smith and co-workers for global peptide proteomic analysis as a means of quantifying relative abundances, and also as a means of distinguishing between near isobaric glutamine and lysine residues (Berger et al. 2002). Ong and colleagues later employed a similar strategy utilizing deuterated leucine and eventually $^{13}C_6$-arginine and termed the methodology stable isotope labeling of amino acids in cell culture (SILAC) as another means of quantifying peptides and proteins (Ong et al. 2002, 2003). Incorporation of ^{13}C and ^{15}N labeled amino acids using the SILAC technique results in co-eluting labeled and unlabeled species and has been widely implemented using isotopically labeled lysine and arginine in conjunction with trypsin digestion for bottom-up proteomics analyses (Ong et al. 2003; de Godoy et al. 2006). Waanders et al. (2007) recently modeled and demonstrated top-down quantification and characterization of the signaling protein Grb2 expressed in *E. coli* using SILAC with $^{13}C_6$-arginine, with accurate quantification at 99 % isotope enrichment. The protein was initially purified off-line using Ni^{2+} affinity chromatography and analyzed using reversed-phase liquid chromatography coupled to an LTQ-Orbitrap mass spectrometer. They also modeled the multiple isotopic envelopes that could result from incomplete incorporation of the heavy isotope amino acid with a 55-kDa protein, but suggested that incomplete labeling could be easily avoided. In our laboratory, we explored the capabilities of intact protein quantification with SILAC and found that even at high levels of stable isotope label incorporation, quantification remained non-trivial. For instance, the isotopic distribution of heavy labeled proteins is certainly broadened at 80 % incorporation of $^{13}C_6$-arginine, as depicted in the labeling of the fungus *Aspergillus flavus* as shown in Fig. 3a. However, even when labels are incorporated at efficiencies approaching 99 % as in the $^{13}C_6$-arginine and $^{13}C_6$, $^{15}N_2$-lysine SILAC labeling of human embryonic stem cells, shown in Fig. 3b, the isotopic distribution is still quite broad and requires special consideration for accurate quantification. This broadening of heavy isotopic distributions is partially attributable to the in vivo conversion of arginine to proline. This modification, in addition to the statistical probability of incorporating unlabeled amino acids, subtracts from the isotopic distribution of the completely labeled species to be quantified (Van Hoof et al. 2007). This is clearly evident in the labeling of lower organisms including fungi like *A. flavus*, which are capable of synthesizing all of their amino acids, but is still observed to a lesser degree in higher organisms including humans. We have developed a simple model for accounting for heavy isotopic distributions when the number of labels and labeling efficiency have been determined (Collier et al. 2008, 2010). In addition to relative quantification, SILAC has also been employed for absolute quantification, in which stable isotope labeled recombinant proteins are generated in expressive cell cultures, purified and accurately quantified, and added to an experimental mixture at a known amount (Hanke et al. 2008).

Other strategies for intact protein quantification

For global quantification of proteins at the peptide level, there are several types of quantitative strategies in addition to metabolic labeling, including enzymatic, chemical, and label-free strategies. There are examples of these types of strategies being applied at the intact protein level, though they have appeared more recently compared to metabolic labeling-based methodologies. Recently, Yan et al. have demonstrated the use of the element coded affinity tags for quantification of intact protein standards. In their study, lysozyme, ribonuclease A, and insulin were reduced, and all free thiol groups were labeled with the addition of a 10-fold excess of 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid-10-maleimidoethylacetamide incorporating either ^{151}Eu of ^{153}Eu and detected using inductively coupled plasma mass spectrometry. While the accurate quantification of tryptic peptides from these proteins was

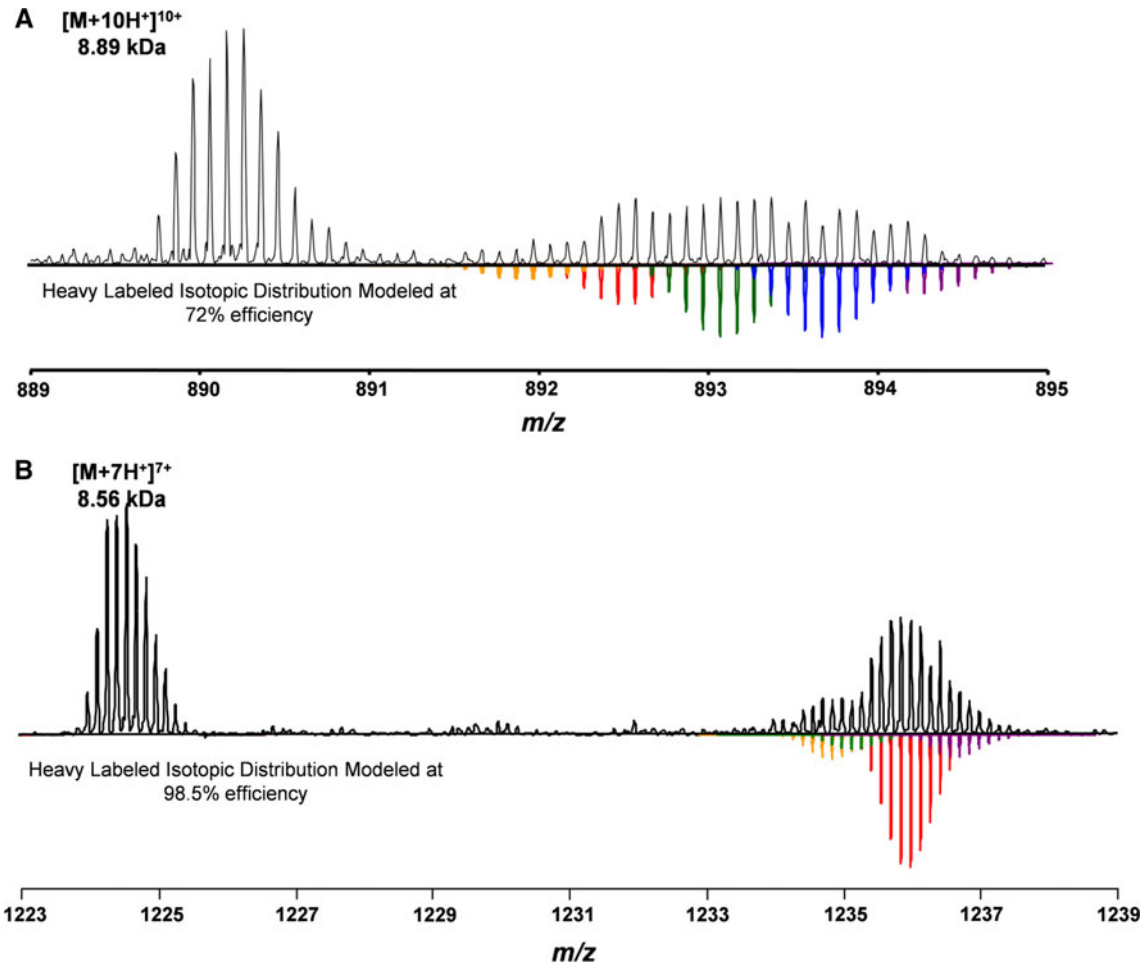

Fig. 3 The success of a quantitative proteomic analysis of intact species using SILAC is reliant upon extensive incorporation of the labeling amino acids. At 72 % incorporation (**a**), the heavy isotopic distribution of a protein from *A. flavus* is scattered across a large *m/z* range and is thus difficult to quantify. When isotopes are incorporated more efficiently at 99 % (**b**), the heavy distribution is more easily identified, though special care must still be taken for accurate quantification. The modeled isotopic distributions are represented as differently colored isotopic distributions on the inverted *y*-axis

hindered by incomplete digestion, the labeled intact proteins were quantified within 6 % with sub-femtomole detection limits (Yan et al. 2010).

Label-free quantification strategies present an attractive alternative to metabolic and chemical labeling, which can be time intensive, cost prohibitive, and not amenable to certain types of biological systems (e.g., prototrophic organisms, inability for cells to be cultured). Two forms of label-free quantification, utilizing integrated ion abundance or spectral counting, have been most widely applied at the bottom-up level. These techniques rely on reproducible instrument methods with a low degree of analytical variability. Muddiman and coworkers established benchmarks of analytical variability for nano-flow liquid chromatography coupled to FT-ICR mass spectrometry including retention time reproducibility, internally and externally calibrated mass measurement accuracy, and ion abundance on the intact species of enriched serum <10 kDa to establish the basis for statistical differentiation of potential biomarkers between healthy and diseased patients (Johnson et al. 2004). This strategy, also known as differential mass spectrometry (Meng et al. 2007), has been recently utilized to quantify intact apolipoproteins in human high-density lipoprotein (HDL) fractions over a fivefold range of expression in a small patient set as a proof of concept for the utility of this technique to top-down proteomics (Mazur et al. 2010).

Because top-down identification and quantification methods have largely lagged behind the development of bottom-up techniques, software for the interpretation of top-down has also been slower to develop. However, algorithms have been developed for the determination of accurate mass from high-resolution mass spectra of intact proteins (THRASH) (Horn et al. 2000) and for the prediction of isotopic distributions (ICR-2LS, PNNL, Richland, WA). Kelleher and colleagues have also recently

developed software for the interpretation of top-down MS/MS data for database searching (Zamdborg et al. 2007). Despite the continuing development of new tools, quantitative top-down methods are largely subject to manual interpretation as the protocols for accurate quantification are still under active development.

Conclusion

The greatest technological challenges to intact protein quantification lie in the separation and detection of intact species, rather than in the strategies to quantify them. In gel-based techniques such as DIGE, spots representing multiple protein isoforms must be enzymatically digested to be extracted from the gel, making it difficult to fully characterize the originating intact protein. Challenges to the analysis of complex protein mixtures in the past included time-consuming off-line separation of samples (Kelleher et al. 1998; Ge et al. 2002; Meng et al. 2002), mass analyzer speed (Siuti and Kelleher 2007), and underdevelopment of database search software tailored to top-down experiments. However, advances in separation technology and methodology (Dillon and Bondarenko 2004; Wang 2005; Tran and Doucette 2008; Vellaichamy et al. 2010), the development of faster hybrid mass analyzer technology such as the LTQ-FT mass spectrometer and LTQ-Orbitrap, and recent innovations in top-down database searching software [e.g., ProSight PTM (Zamdborg et al. 2007)] have created the potential for more widespread utilization of top-down proteomics techniques. Top-down mass spectrometry is still evolving to incorporate quantitative methodologies that have become a commonality with bottom-up analyses. Combined with its powerful capability of molecular characterization, quantitative top-down strategies have the potential to contribute to proteomics on a large scale.

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