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Absolute Quantification of Proteins in Solutions and in Polyacrylamide Gels by Mass Spectrometry

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A combination of nanoelectrospray tandem mass spectrometry and ^{18}O-labeled peptide internal standards was applied for the absolute quantification of proteins from their in-solution and in-gel tryptic digests. Although absolute quantification from in-solution digests was accurate, we observed that in-gel digestion compromised the quantification accuracy by affecting the recovery of individual peptides and, therefore, the provided estimates might be strongly influenced by the selection of reference peptides. Under optimized experimental conditions, it was possible to provide a semiquantitative estimate of the absolute amount of gel separated proteins within better than 50% error margin.

Advances in mass spectrometry, database-searching algorithms, and genetic engineering have improved our understanding of how individual proteins assemble in complexes and create a functional framework of prokaryotic and eukaryotic proteomes;[1−4] however, it has also become apparent that the organization of a proteome-wide interaction network is exceedingly complex. On average, every fourth protein in a eukaryotic proteome might be shared between different protein complexes.[4] The protein function often remains elusive in a network context, since it is often unclear if a protein represents a stoichiometric "core" of the complex or it is a transient substoichiometric interactor that "hyperlinks" individual complexes.[5] Although many fully functional protein complexes have been isolated by immunoaffinity chromatography,[6] it is still unclear if their stoichiometry is stringent or they are merely clusters of associated proteins in which the stoichiometric ratio between subunits may vary. To understand the role of proteins within a network, their interactions should be characterized quantitatively.

Mass spectrometry has developed into a powerful tool for the quantification of proteins because of its high sensitivity, speed, and specificity that have enabled quantification of individual proteins in mixtures.[7−9] Relative (or comparative) quantification usually determines relative changes of the amount of a given protein in experimental and control samples. Relative amounts of many proteins can be compared in parallel, thus providing a quantitative overview of the dynamically altered proteome. Proteins can be metabolically labeled with stable isotopes by growing cells in isotopically enriched media. Experimental and control cell pools are then mixed, and proteins of interest can be further enriched by sophisticated analytical procedures, which would not affect the accuracy of the analysis, since isotopically labeled and native proteins will likely behave similarly.[10,11] Alternatively, protein mixtures recovered from experimental and control cells can be separately treated with isotopically labeled and unlabeled chemical probes.[12] The derivatized protein pools are then mixed and digested with enzymes, and modified peptides are enriched by affinity chromatography and quantified by LC−MS/MS. In both approaches, proteins are quantified by comparison of intensities or peak areas of the isotopically labeled and native forms of the same peptide, and the protein amount is averaged if several pairs of peptides originating from the same proteins are analyzed. Both approaches enable accurate relative comparison of the amount of the same protein, no matter how many different proteins were analyzed in parallel; however, it is not possible to quantify the relative amount of different proteins, since the response of a mass spectrometer strongly depends on the amino acid composition and sequence of analyzed peptides.

Another analytical approach determines the absolute amount of the analyzed protein, either in moles or in grams per cell or per purification, and therefore, the content of different proteins present in the sample can be directly compared. Being more direct, the absolute quantification is also more technically demanding, since it is important that peptides with sequences that are almost identical to sequences of peptides from the quantified analyte are employed as internal standards.

If a stock solution of the target protein is available, it can be digested with trypsin and the digest further analyzed by LC−MS or LC−MS/MS. The combined area of peaks of its tryptic peptides can be used to estimate its amount, even in a heavy mixture with

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other proteins.[13] On-line sequencing of eluted peptides also produces multiple fragment ions for each peptide, thus increasing the specificity of its identification and the accuracy of quantification.[14]

Synthetic peptides, which either incorporate amino acids enriched with stable isotopes or are labeled by stable isotopes in a chemical or enzymatic reaction are commonly employed as internal standards for absolute quantification. Isotopicaly labeled peptides have chemical properties almost identical to properties of the corresponding peptides from the analyte, but because of the difference in their intact masses or masses of fragment ions, they can be readily distinguished by MS or MS/MS methods. The absolute concentration of a protein can be calculated from a ratio of intensities of signals derived from the analyte and from the internal standard.[15] Tryptic peptides derived from the analyzed proteins and synthetic isotopically labeled internal standards were employed in absolute quantification of proteins in-solution[16,17] and, recently, in-gel.[18]

Isotopically labeled internal standards can also be obtained by digesting a stock solution of the protein of interest with trypsin in a buffer containing $H_2{}^{18}O$.[19] Upon digestion, ^{18}O atoms are incorporated into C-terminal carboxyl groups of tryptic peptides. Peptides with C-terminal lysine residues mostly incorporate one ^{18}O atom, whereas peptides with C-terminal arginine residues typically incorporate two atoms, and their masses are shifted from the masses of unlabeled peptides by 2 and 4 Da, respectively.[20] ^{18}O-labeled digest is then spiked into a digest of the target protein, and labeled peptides are employed as internal standards for protein quantification.[21] Although isotopic clusters of ^{18}O-labeled peptides partially overlap with isotopic clusters of unlabeled peptides, simple deconvolution algorithms enable accurate determination of peak areas of the reference peptide and the analyte.[22,23] Arginine-containing peptides usually dominate in MALDI spectra of protein digests,[24] which together with favorable 4 Da mass shift makes them preferred standards for the ^{18}O-based quantification; however, the possibility of variable modification of certain amino acid residues, such as oxidation of methionine or tryptophane residues, or desamidation of asparagine residues, or the presence of internal cleavage site(s), should also be considered when selecting appropriate reference peptides. Under certain reaction conditions, the yield of peptides rendered by in solution digestion of a target protein might be close to 100%, and therefore, the protein concentration is directly proportional to the average concentration of individual tryptic peptides in the digest. The method is relatively simple, robust and directly compatible with both MALDI- and ESI-MS.

Although a few absolute quantification approaches have been developed, it is still unclear if a polyacrylamide gel matrix affects the yield of peptides from different proteins in a different way and whether the quantification in-solution and in-gel is expected to produce different estimates of the protein amount. It is equally important to determine which experimental conditions, such as enzyme concentration, digestion temperature, extraction solvent, etc., would enable the most robust absolute quantification over a wide range of proteins with different physical properties. To this end, we performed the comparative quantification of several model proteins from their in-solution and in-gel digests. We observed that the digestion of proteins embedded into a polyacrylamide matrix affects the recovery of peptides, and therefore, the results may strongly depend on what peptides were selected as a references for the quantification. We also determined the experimental conditions that enabled a robust semiquantitative estimate of the absolute amount of gel-separated proteins within ~50% error margin.

EXPERIMENTAL SECTION

Materials and Reagents. All chemicals were purchased from Sigma (Sigma Chemicals, St. Louis, MO) and were of analytical grade, unless otherwise noted. Concentration of stock solutions of standard proteins bovine serum albumin (BSA), budding yeast glucose-6-phosphate dehydrogenase (GPD), bovine carbonic anhydrase (CAH), and equine heart myoglobin (MYO) and synthetic peptides was determined by amino acid analysis performed in the laboratory of Dr. P. Hunziker at the University of Zürich. For further use, 95% $H_2{}^{18}O$ (Cambridge Isotopic Laboratories, MA) was purified by microdistillation, as described.[25] Modified porcine trypsin was purchased from Promega (sequencing grade modified trypsin, Catalog No. V5111, Mannheim, Germany). 1-Cyano-4-hydroxycinnamic acid (a matrix for preparing MALDI probes) was purchased from Bruker Daltonik GmbH (Bremen, Germany). Isotopically labeled AQUA peptide standards of horse myoglobin were kindly provided by Drs. S. A. Gerber and S. P. Gygi (Harvard Medical School, Boston, MA).

Instruments and Software. Tandem mass spectrometry experiments were performed on a modified QSTAR Pulsar *i* quadrupole time-of-flight mass spectrometer (MDS Sciex, Concord, Ontario, Canada) equipped with a nanoelectrospray ion source (Proxeon Biosystems A/S, Odense, Denmark).

To acquire MS/MS spectra of internal standards, precursors were selected by the analytical quadrupole Q1 within a m/z window of ~4 Da for ^{18}O-labeled and 2 Da for AQUA peptides to ensure unperturbed transmission of the isotopic clusters of doubly charged peptide ions. Collision energy was adjusted manually for the highest yield of relevant fragment ions. Ion current was controlled to avoid saturating the detection system, and peak intensities were always lower than 700 counts/s (cps). Spectra were processed using Analyst QS SP6 (MDS Sciex, Concord,

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Ontario, Canada) and ACD/SpecViewer 5.04 (Advanced Chemistry Development, Toronto, Ontario, Canada).

Protein quantification by MALDI-TOF was performed on a Reflex IV mass spectrometer equipped with a Scout 384 ion source (Bruker Daltonics GmbH, Bremen, Germany). Spectra were processed by XMass 1.5.1 (Bruker Daltonics, Bremen) and ACD/SpecViewer 5.04 (Advanced Chemistry Development, Toronto, Canada).

In-Gel Digestion of Standard Proteins. Proteins were separated by one-dimensional SDS-polyacrylamide gel electrophoresis on a Bio-Rad Mini-Protean II system using 12% polyacrylamide gels of 1-mm thickness. Aliquots of stock solutions of proteins prepared in 1% formic acid were diluted with a sample buffer to a final concentration ~1.0 or 3.0 μM, and then 5 μL of this solution was loaded onto a gel. Upon electrophoresis, proteins were visualized by Coomassie Brilliant Blue R250 staining (Serva Electrophoresis GmbH, Heidelberg, Germany). Protein bands were excised, cut into 1 mm^3 cubes, put into 0.65-mL PCR microtubes (Roth, Karlsruhe, Germany), and in-gel digested using modified trypsin as described previously.[22] Destained, washed, and dehydrated gel pieces were rehydrated for 60 min in a 0.5 or 10.0 μM solution of the modified trypsin in 50 mM ammonium bicarbonate buffer at 4 °C. The digestion was carried out for 720 min at 37 or 58 °C and stopped by the addition of 10% formic acid.

In-Solution Digestion of Standard Proteins. A 10-μL aliquot of a stock solution were dried in a vacuum centrifuge and redissolved in 40 μL of either $H_2{}^{16}O$ or $H_2{}^{18}O$, depending on the particular experiment. A 1-μL aliquot of unmodified bovine trypsin was added to achieve an enzyme-to-substrate ratio of 1:5, followed by 0.5 μL of 2.5 M ammonium bicarbonate. Trypsin and ammonium bicarbonate were dissolved in ^{18}O-water to produce isotopically labeled standards. Digestion was performed at 37 °C for 720 min and stopped with 10% formic acid.

In further experiments, standard proteins were quantified using the following reference tryptic peptides: BSA: YLYEIAR (B1), KVPQVSTPTLVEVSR (B2), LVNELTEFAK (B3), LGEYGFQNALIVR (B4), DAFLGSFLYEYSR (B5); GPD: IDHYLGK (G1), VQPDAAVYLK (G2), WEGVPIMMR (G3), FGNQFLNASWNR (G4), NTVISVFGASGDLAK (G5); CAH: VLDALDSIK (C1), QSPVNIDTK (C2); and MYO: ALELFR (M1), LFTGHPETLEK (M2).

Protein Quantification In-Solution. Digestion of proteins in solution was performed as described above with a few modifications applied in the specific experiments.

Linearity of the Calibration Curve. The stock of the in-solution digest of BSA was diluted to 13.5, 27.0, 54.0, 108.0, and 270.0 μM concentration. ^{18}O-labeled BSA tryptic peptide mixture (108.0 μM, internal standard) was spiked into the samples prior to measurements. Spiked protein samples were further diluted 70 times with methanol/10% formic acid 3:2 (v/v) to reach the range of peptide concentration 0.2−3.9 μM and loaded into a spraying needle. The concentrations of three tryptic peptides, B1, B4, and B5, were determined for each dilution as described previously,[22] and the results were averaged.

Completeness of In-Solution Digestion. In-solution digest of 3.8 μM BSA in $H_2{}^{18}O$ was spiked with a known concentration of three synthetic standard tryptic peptides, B1, B4, B5 (Eurogentec, Seraing, Belgium), at 1.5, 1.2, and 1.5 μM, respectively. Isotopic clusters of unlabeled and ^{18}O-labeled peptides were deconvoluted as described below. The actual concentration of ^{18}O-labeled digestion products was calculated from the ratio of the intensities of labeled and unlabeled forms and compared with the start concentration of BSA.

Absolute Quantification of Proteins In-Solution. Mixtures containing BSA and GPD in different molar ratios, 1.3 μM BSA and 1.3 μM GPD, 3.8 μM BSA and 1.3 μM GPD, and 1.3 μM BSA and 4.0 μM GPD, were digested by trypsin, and each sample was spiked with an aliquot of ^{18}O-labeled peptide standard containing 2.1 μM BSA and 1.8 μM GPD. Proteins were quantified using five reference tryptic peptides for BSA (B1−B5) and five reference tryptic peptides for GPD (G1−G5). Oxidized forms of methionine- and tryptophane-containing peptides were not detected.

Absolute Quantification of Proteins In-Gel. Mixtures of 6.5 pmol of BSA and 6.7 pmol of GPD, 6.5 pmol of BSA and 20.0 pmol of GPD, and 18.8 pmol of BSA and 6.7 of pmol GPD were loaded onto a polyacrylamide gel, and bands were resolved by electrophoresis. Protein bands were excised, digested in-gel with trypsin, and the recovered mixture of tryptic peptides was spiked with ^{18}O-labeled internal standard, 2.1 and 1.8 μM, respectively. The yield of five tryptic peptides of BSA (B1−B5) and five tryptic peptides of GPD (G1−G5) was determined as described above; yields were averaged and compared with the amount of proteins loaded on a gel.

In-Gel Quantification and Optimization of the Sample Preparation. Pre- and postdigestion sample processing was evaluated as follows:

Extraction of In-Gel Digestion Products. In-gel digest of 6.7 pmol of GPD was extracted by 5% formic acid/acetonitrile as described previously.[26] In a parallel experiment with exactly the same loading of GPD, the supernatant of the in-gel digest was recovered from the tube with gel pieces. The gel pieces were washed with a volume of water equal to the volume of the recovered supernatant. Washing water was recovered and combined with the supernatant. Both samples (the formic acid/acetonitrile extract and the supernatant) were dried down in a vacuum centrifuge, spiked with a 1-μL aliquot of 1.8 μM ^{18}O-labeled digest (internal standard), and quantified. The yield of four GPD tryptic peptides (G2−G5) was determined and averaged.

Effect of the Solid-Phase Extraction. In-solution digests of two model proteins, 1.3 μM BSA and 4.0 μM GPD, were extracted, dried down, redissolved in 5% formic acid, and split into equal volumes. One-half of the sample was subjected to SPE (solid phase extraction) cleanup on an in-house-made microcolumn packed with POROS R2 reversed-phase material (see column preparation details below) before mass spectrometric data acquisition, whereas the second half of the sample was analyzed directly without cleanup. The yield of five tryptic peptides of BSA and five peptides of GPD was quantified as described above.

Quantification by MALDI vs Quantification by NanoESI. Two aliquots of an in-gel digest of 6.5 pmol of BSA were quantified by nanoESI and by MALDI. In the MALDI-MS experiment, the yield was quantified using three peptides: B1, B4, and B5. In the nanoESI experiment, B5 peptide was not abundant, and therefore,

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B1 and B4 peptides were used for the quantification. Details of the quantification procedures are provided below.

Effect of Unbiased Loss of Peptides during In-Gel Digestion. Twenty picomoles of GPD was in-gel-digested overnight at 37 °C, and the hydrolysis was stopped with 10% formic acid. The acidified digest was spiked with 1 μL of 1.8 μM of ^{18}O-digest of GPD (internal standard), and the mixture was incubated at 37 °C for another 8 h. A separate aliquot of the ^{18}O digest of GPD was acidified and incubated in parallel with the sample as a control for possible exchange of ^{18}O atoms to ^{16}O atoms in water. Subsequent MALDI-MS analysis of the control suggested that no back-exchange occurred under these experimental settings. The mixture of in-gel-digested model protein and internal standard was extracted and analyzed by nanoESI QqTOF-MS. The digestion yield was quantified using four tryptic peptides (G2−G5), as described above.

Effect of Enzyme Concentration and Digestion Temperature. Bands containing 6.5 pmol of BSA were digested in-gel at 37 °C in the buffers containing 0.5 and 10 μL of trypsin. In addition, bands containing 6.5 pmol of BSA, 6.7 pmol of GPD, and 9.0 pmol of CAH were digested in-gel using a higher concentration of trypsin (10 μM) and at elevated temperature (58 °C). The digests were spiked with corresponding ^{18}O-labeled internal standards. Peptide recovery was quantified and compared with the loaded amount of proteins. To evaluate the peptide yield, five tryptic peptides of BSA (B1−B5), four peptides of GPD (G2−G5), and two peptides of CAH (C1, C2) were used.

Effect of the Internal Standard. A Coomassie-stained band containing 5.2 pmol of MYO was digested in-gel in 10 μM trypsin at 37 °C. In one experiment, ^{18}O-labeled tryptic peptides were used as internal standards for the quantification. In the second experiment, isotopically labeled AQUA peptides ALEL*FR, LFTGHPETL*EK, where L* stands for leucine with one ^{15}N and six ^{13}C atoms,[18] were applied. To evaluate the digestion yield, the recovery of two peptides, M1 and M2, was determined.

Quantification by NanoESI MS/MS. Products of in-gel digestion were extracted using water, 10% formic acid, and acetonitrile, as described previously.[25] The recovered extracts were pooled together and dried down in a vacuum centrifuge. The dried extract was redissolved in 10 μL of 10% formic acid and desalted/preconcentrated on a homemade microcolumn containing POROS R2 resin to a final volume of 2 μL of methanol/10% formic acid 3:2 (v/v).[4,25] A mixture of ^{18}O-labeled peptides (internal standard) was added directly onto the column and eluted with the preconcentrated samples (unless specified otherwise). A metal-coated needle (Proxeon Biosystems, Odense, Denmark) was used to electrospray the sample. Each quantification experiment was performed in duplicate, with three sets of 100 spectra acquired and averaged per each quantified peptide. Spectra were smoothed, and peaks were automatically designated using a centroid peak-picking algorithm. Spectra presented as peak intensity (in counts) vs time of flight (in microseconds) were exported as ASCII files and imported into the ACD/SpecViewer program for graphical integration and calculation of peak areas.

Quantification by MALDI-MS. Sample preparation and data acquisition using MALDI-MS were performed as described previously.[22] MALDI spectra presented as peak intensity (in absolute units) vs time of flight (in microseconds) were exported as ASCII files, and peak areas were calculated by graphical integration in the ACD/SpecViewer program.

Experimental Errors. Relative error of pipetting was 6%, as determined in a separate experiment. Errors of the amino acid analysis were 9% for BSA and 19% for GPD, as calculated from the data reports. Average error of MS/MS quantification was 5%. The overall experimental errors of determined concentrations of BSA and GPD were 12 and 21%, respectively.

Employed Quantification Approaches. *Absolute Quantification Using AQUA Peptides*. A quantified protein was digested in a buffer containing $H_2{}^{16}O$ water and a known amount of AQUA peptide standards. The quantified peptides and corresponding AQUA standards were fragmented by nanoESI QqTOF-MS/MS. In the MS/MS spectra of the quantified peptide and AQUA standards, y-ions[27] having m/z higher than the m/z of doubly charged precursors were matched, and areas of their monoisotopic peaks were determined. The amount of peptides present in the mixture was calculated from the ratios of the areas of the monoisotopic peaks of matching fragment ions.

Absolute Quantification Using ^{18}O-Labeled Peptides. A quantified protein was digested in a buffer containing $H_2{}^{16}O$ water. A sample of the same protein with known concentration was digested in $H_2{}^{18}O$ water, rendering tryptic peptides labeled with one or two ^{18}O atoms at their C-termini. The known amount of a mixture of ^{18}O-labeled peptides (internal standard) was spiked into a digest of the quantified protein. A full isotopic cluster of a mixture of unlabeled and ^{18}O-labeled peptide was selected by Q1 quadrupole and fragmented, and pairs of y-ions were matched as described above. The amount of peptides present in the mixture was calculated from the ratio of the areas of monoisotopic peaks of the unlabeled fragment, and the deconvoluted area of the peak corresponding to the 2 × ^{18}O-labeled fragment.[22,23]

Absolute Quantification Using Unlabeled Synthetic Peptides. A quantified protein was digested in $H_2{}^{18}O$, rendering mono- and doubly ^{18}O-labeled peptides. This mixture was spiked with the known amount of unlabeled synthetic peptides. The amount of a quantified protein was calculated as described above.

Deconvolution of Isotopic Clusters of ^{18}O-Labeled Peptides. A typical MS/MS spectrum used in the protein amount determination is presented in Figure 1. In all cases, the amount of an individual digestion product, m_{sample}, was calculated from the amount of the internal standard, m_{istd}, and the ratio of the averaged areas of the monoisotopic peak of the unlabeled peptide, A_{sample}, and of the isotopically labeled internal standard, A_{istd}.

$$m_{\text{sample}} = m_{\text{istd}} \frac{A_{\text{sample}}}{A_{\text{istd}}}$$

If the standard was ^{18}O-labeled, the latter area was calculated by deconvoluting the isotopic cluster.[22]

$$m_{\text{sample}} = \frac{A_{\text{sample}}}{A_{\text{double}} + A_{\text{single}}(1 - f_3) + A_{\text{sample}}(f_3^{\,2} - f_5 - f_3)} m_{\text{istd}}$$

where A_{single} and A_{double} are the areas of the isotopic peaks spaced

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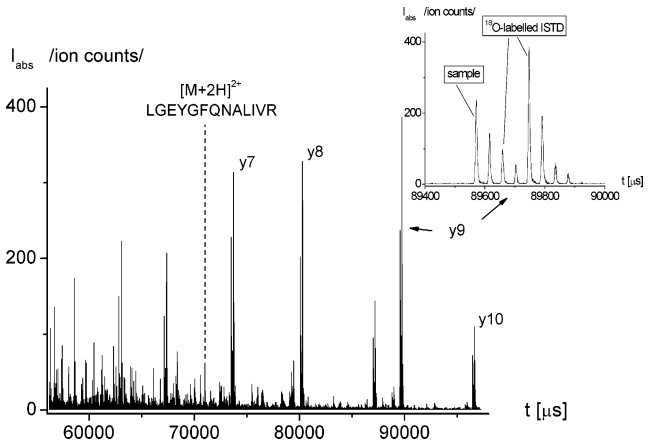

Figure 1. MS/MS spectrum of an isotopic cluster of a doubly charged ion of ^{18}O-labeled and unlabeled BSA tryptic peptide LGEYGFQNALIVR (B4). Inset: isotopic cluster of y_9 fragment ion, comprising unlabeled, singly, and doubly ^{18}O-labeled peptide forms. For accurate quantification, it is important that the spectrum is represented in intensity vs time of flight coordinates. If plotted against *m*/*z*, the number of acquired bins per detected peak (and, consequently, the peak area) will depend on its *m*/*z*.

from the monoisotopic peak of the unlabeled peptide by 2 and 4 Da, respectively. Coefficients f_3 and f_5 are the calculated ratios of the intensity of, respectively, third (+2 Da) and fifth (+4 Da) isotopic peaks to the intensity of the monoisotopic peak of the unlabeled peptide; f_3 and f_5 were calculated for each peptide using the MS-Isotope program from ProteinProspector software (UCSF mass spectrometry facility), available at http://prospector.ucsf.edu.

To determine the concentration of individual tryptic peptides, areas of peaks of typically three to five y-ion fragments were determined. The data presented the average of three parallel runs.

To calculate the amount of proteins, the amount of two to five peptides was determined and averaged. The data presents the average of three parallel runs.

Standard deviation of the mean was calculated for each data set.

RESULTS AND DISCUSSION

The Quantification Method. In mass spectrometry-based quantification, the protein amount is usually not measured directly, but is rather inferred from the experimentally determined amount of peptides rendered by digestion of the target protein in-solution or in-gel; however, chemical and physical properties of proteins are rather diverse, and therefore, the recovery of peptides might vary considerably. Protein quantification via ^{18}O labeling encompasses almost any detectable peptide, since internal standards (^{18}O-labeled peptides) can be directly obtained by digesting a stock solution of the protein of interest; however, for better consistency of measurements, it was important to ensure that (a) mass spectrometric quantification relied on a linear calibration curve and (b) digestion of the stock solution of the protein standard was complete.

We tested the linearity of the instrument response by analyzing the series of samples prepared by the serial dilution of an in-solution digest of BSA, which were spiked with equal amount of a mixture of ^{18}O-labeled BSA peptides produced in a separate experiment. We plotted the ratio of peak areas of the sample and the internal standard against the ratio of concentrations of the sample and of the internal standard. The slope value of the calibration line was 1.0 ± 0.1, the ordinate intercept was 0.01, and the correlation coefficient 0.995 (data not shown). For practical reasons, in further experiments, we maintained the ratio of concentrations of the sample and the internal standard within a range of 1:10 to 10:1, whereas the absolute concentration of the analyte was within a range of 50 nM to 5 μM.

Next, we tested if the in-solution digestion of BSA in a $H_2{}^{18}O$ buffer was complete and all digestion products had efficiently incorporated ^{18}O atoms.[28] To this end, we performed a "reversed quantification", that is, a known amount of BSA was digested in $H_2{}^{18}O$ buffer and spiked the digest with a known amount of unlabeled synthetic peptides having the same sequence as the reference tryptic peptides from BSA. The comparison of peak intensities suggested that the concentration of reference peptides was close to the one expected from the concentration of the intact protein, on average 103 ± 3%, and was within an experimental error margin of amino acid analysis (data not shown).

Absolute and Relative Quantification of Proteins In-Solution and In-Gel. Next, we investigated if the approach provides accurate absolute and relative quantification of proteins from in-gel and in-solution digests. Stock solutions of BSA and GPD were mixed to yield three samples with different molar ratios between the proteins, ~1:1, 3:1, and 1:3 (BSA/GPD). Each of these samples was digested in-solution with trypsin. In another experiment, 5 μL of the same protein mixtures was loaded on a gel, and bands were separated by electrophoresis, excised, and in-gel-digested, and recovered peptides were quantified using spiked ^{18}O-labeled standards. The determined absolute concentrations of proteins (and hence, their molar ratio) matched corresponding calculated values within the error margin if proteins were digested in-solution (Figure 2A); however, the yield of in-gel digestion of these proteins was rather low and enabled no accurate absolute or relative estimates (Figure 2B). Furthermore, we observed that yields of individual peptides varied strongly in in-gel digests (Figure 3), leading to a high RSD of the measured amounts of proteins. The yield of five BSA peptides was similar (Figure 3B); however, some GPD peptides were strongly underrepresented in the in-gel digest, as compared to the in-solution digest (Figure 3A). We further checked if a peptide with the lowest yield (G3, WEGVPIMMR) was lost because of oxidation of tryptophane or methionine residues, but almost no ions of corresponding multi-oxidized forms were detected by MALDI-MS or nanoESI-MS. Relatively large dispersion of peptide yields pointed to possible misinterpretation of in-gel quantification of proteins, since it could be affected by biased selection of reference peptides.

In-Gel Quantification and Sample Preparation Methods. Under conventional conditions of in-gel digestion, the yield of tryptic peptides is, first, protein-dependent, and second, it varies strongly for different peptides that originate from the same protein. At the same time, digestion of proteins in-solution enabled their robust quantification. We reasoned that two major factors (or their combination) might be responsible for reduced and biased yields of peptides in in-gel digests. First, the efficiency of in-gel digestion might be lower as compared to the digestion in-solution and might

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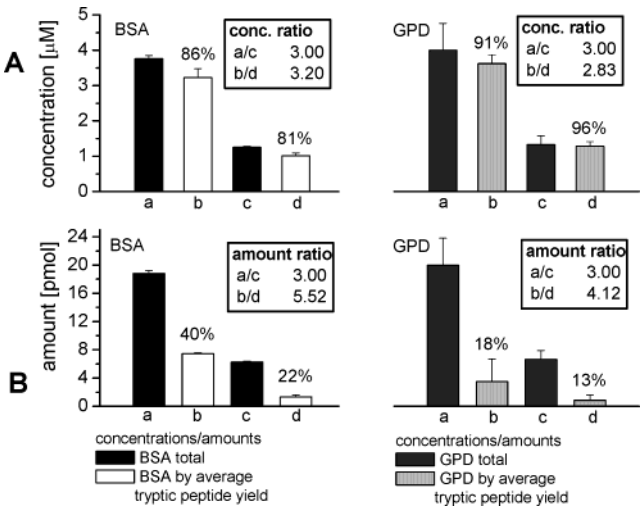

Figure 2. Comparison of peptide yields for in-solution (A) and in-gel (B) digestion of two different concentrations and amounts of BSA and GPD. Concentrations of proteins in the in-solution digests (panel A): 3.8 (a) and 1.3 (c) μM for BSA and 4.0 μM (a) and 1.3 (c) for GPD. For in-gel digestions (panel B), the amounts of proteins loaded on a gel were 18.8 (a) and 6.3 (c) pmol for BSA and 20.0 (a) and 6.7 (c) pmol for GPD. The digestion yield averaged for all quantified peptides is presented in percent. The protein concentrations determined by in-solution digests were determined in three independent experiments by digestion of mixtures of BSA and GPD present in various molar ratios.

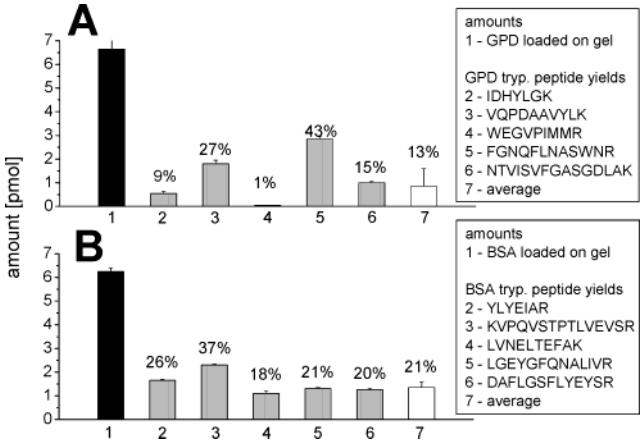

Figure 3. Yields of individual tryptic peptides of in-gel-digested GPD (A) and BSA (B) under conventional digestion conditions: trypsin concentration 0.5 μM, digestion temperature 37 °C. The peptide yield is presented in percentage of the loaded amount of proteins: 6.3 pmol for BSA; 6.7 pmol for GPD.

render a different set of digestion products. Second, biased losses of peptides might occur during postdigestion sample processing of in-gel digests, for example, during the extraction of peptides from a polyacrylamide matrix. We further focused on the latter reason and set out to determine if any particular step in the sample preparation and quantification protocols might cause biased loss of peptides from in-gel digests.

We first checked how sample cleanup by a solid-phase extraction affected the yield of various peptides. Although it is known that small or hydrophilic peptides could be lost in a single-stage reversed-phase purification,[29,30] such loss would equally affect quantified (unlabeled) and internal standard (^{18}O-labeled) peptides if their mixture is directly applied onto the column. We spiked

Table 1. Peptide Yields from the In-Solution Digests of BSA and GPD Determined by NanoESI with and without a Cleanup Step

SPE cleanup	BSA		GPD			
no	c_{calc}[a] [μM]	1.3	c_{calc} [μM]	4.0	R_{calc}[c]	3.1
	c_{exp}[b] [μM]	1.0	c_{exp} [μM]	3.6	R_{exp}[d]	3.6
	SD[g]	0.1	SD	0.3	Δ[e]	0.5
	RSD [%][h]	10	RSD [%]	8	rel Δ [%][f]	16
yes	c_{calc} [μM]	1.3	c_{calc} [μM]	4.0	R_{calc}	3.1
	c_{exp} [μM]	1.1	c_{exp} [μM]	3.7	R_{exp}	3.4
	SD	0.1	SD	0.9	Δ	0.3
	RSD [%]	9	RSD [%]	24	rel Δ [%]	10

[a] Protein concentration as determined by amino acid analysis of the stock solutions. [b] Protein concentration as determined by MS. [c] Ratio of BSA and GPD concentrations (GPD/BSA) calculated from their c_{calc} values. [d] Ratio of BSA and GPD concentrations (GPD/BSA) calculated from their c_{exp} values. [e] Difference between R_{exp} and R_{calc}. [f] Relative difference between R_{exp} and R_{calc}, in percent. [g] Standard deviation of c_{exp}. [h] Relative standard deviation of c_{exp}.

aliquots of the in-solution digest with ^{18}O-labeled internal standard, purified the mixture on a reversed-phase resin, and quantified the eluted peptides by nanoESI. In parallel experiments, the same mixtures were analyzed by nanoESI directly. We observed that under the employed experimental conditions, the cleanup step did not substantially affect the quantification of both proteins (Table 1).

Further, we checked if the extraction of peptides from the gel pieces by 5% formic acid and acetonitrile might result in a preferential enrichment of certain peptides, whereas other peptides were lost. To this end, we extracted the series of in-gel digests of GPD. In parallel experiments, only the supernatant (the digestion buffer, surrounding gel pieces in the test tube) was recovered, but no further extraction of gel pieces by formic acid or organic solvents was performed. We found that the extraction did not increase the average yield of GPD peptides substantially and that the dispersion of yields of individual peptides remained high, thus resulting in poor quantification accuracy (Table 2).

We also considered the possibility that several peptides might be preferentially absorbed by a gel matrix and were not released into the supernatant so that such losses would not be compensated by spiking the internal standards. ^{18}O-labeled peptides cannot be added into a digestion buffer directly, since trypsin would catalyze back-exchange of ^{18}O atoms to ^{16}O atoms in carboxyl groups of C-terminal arginine residues. Therefore, we acidified an in-gel digest of GPD with 10% formic acid and spiked ^{18}O-labeled internal standard into the sample. Further, we incubated the sample for 8 h at 37 °C to let the ^{18}O-labeled peptide standard diffuse into gel pieces, as if internal standard would have been present in the sample right from the start of digestion. After the incubation, the sample was extracted, and the peptide yields were determined (Figure 4). Spiking the internal standard into the mixture improved the apparent yield of GPD peptides, with the yield of the most underrepresented peptide increased by almost 10-fold; however, the average yield was still well below the one expected from the known concentration of GDP (41%). It did not reach the

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Table 2. Yields of GPD Peptides Determined by NanoESI with and without Extraction of Gel Pieces

GPD	extracted		not extracted	
trial 1	n_{calc}[a] [pmol]	6.7	n_{calc} [pmol]	6.7
	n_{exp}[b] [pmol]	2.0	n_{calc} [pmol]	3.5
	SD[c]	1.5	SD	3.0
	RSD[d] [%]	75	RSD [%]	86
	recovery [%][e]	31	recovery [%]	54
trial 2	n_{calc} [pmol]	6.7	n_{calc} [pmol]	6.7
	n_{exp} [pmol]	2.5	n_{exp} [pmol]	2.5
	SD	2.0	SD	1.5
	RSD [%]	80	RSD [%]	60
	recovery [%]	38	recovery [%]	38

[a] Protein amount loaded on gel originated in amino acid analysis of the stock solutions. [b] Protein amount recalculated from protein amount determined by nanoESI. [c] Standard deviation of n_{exp} estimation. [d] Relative standard deviation of n_{exp} estimation. [e] Percentage of tryptic peptide recovery.

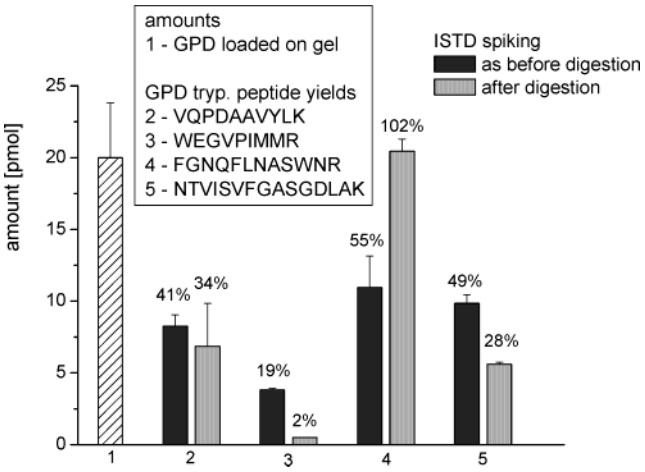

Figure 4. Internal standards (^{18}O-labeled peptides) were spiked into the in-gel digest of GPD before and after digestion. Bars represent 1, total amount of GPD loaded on a gel; 2−5, yield of GPD tryptic peptides G2−G5, respectively. Digestion yield is presented in percentage of the loaded amount of protein (20 pmol).

yield of the in-solution digestion, and the variability in the recovery of individual peptides remained substantial.

This prompted us to speculate that low recovery of the digestion products is most likely determined by the kinetics of in-gel digestion of a protein substrate, rather than by biased losses that occurred during postdigestion handling of the sample. We note, however, that this conclusion is only supported by the observed intensities of a small number of the most intense peptide peaks that were most suitable for the subsequent quantification, rather than by a comprehensive interpretation of peptide mass fingerprints.

Internal Standards and Mass Spectrometric Detection. Next, we asked if absolute quantification by mass spectrometry depends on employed internal standards and on the method of mass spectrometric detection of reference peptides. Further prompting this question, the successful absolute quantification of gel-separated myoglobin by LC−MS/MS and synthetic peptide standards containing isotopically labeled leucine residues was recently reported by Gerber et al.[18] We therefore quantified the in-gel digest of 5.2 pmol myoglobin by nanoESI using the same set of synthetic peptides and the same digestion conditions as described by Gerber et al. In a parallel experiment, the same amount of gel-separated myoglobin was quantified using ^{18}O-labeled peptides, and the results were compared. Quantification based on ^{18}O-labeled peptides estimated the amount of myoglobin at 5.3 ± 0.6 pmol, whereas using AQUA peptides, it was determined as 4.7 ± 0.7 pmol. We, therefore, concluded that under the chosen experimental conditions, the results of quantification experiments did not depend on the type of employed internal standards.

We further considered if ^{18}O quantification would be affected by the employed mass spectrometric method. Series of in-gel digests of bands, each containing 6.5 pmol of BSA were analyzed by MALDI-MS and by nanoESI. The average yield of tryptic peptides determined by MALDI-MS and nanoESI-MS was 43 ± 13% and 35 ± 5%, respectively. Thus, both methods produced consistent results; however, the nanoESI method was more accurate and allowed relatively straightforward quantification in simple protein mixtures. Longer acquisition time provided reliable ion statistics for many fragment peaks acquired from multiple precursor ions. Thus, accurate calculation of peak areas and subsequent quantification were simplified; however, it has also become apparent that for quantifying protein mixtures of higher complexity, LC−MS/MS capabilities are indispensable.

Increasing the Peptide Yield of In-Gel Digestion for Absolute Quantification. The outcome of the above experiments prompted us to conclude that the completeness of in-gel digestion is a major limiting factor in accurate absolute quantification of gel-separated proteins. We previously reported[22] that higher concentration of methylated autolysis-resistant trypsin (>5 μM) and elevated temperature (58 °C) resulted in higher yield of in-gel digestion. The protocol (termed ADP for accelerated digestion protocol) was successfully applied for the identification of gel-separated proteins and provided high-quality peptide mass fingerprints after 30 min of in-gel digestion. We tested if the reaction conditions, similar to the ones used in ADP, would also help us to provide robust absolute quantification.

BSA, GPD, and CAH were digested in 10 μM modified trypsin at 58 °C. Although we observed a substantial increase in the average yield of digestion products, the cleavage was still incomplete, reaching ∼70% for BSA, 40% for GPD, and 80% for CAH, and reproducibility was rather poor because abundant background of trypsin autolysis products compromised the quality of nanoESI quantification. Therefore, we concluded that under the current settings, it was only possible to provide a rather rough estimate of the absolute amount of gel-separated proteins within ∼50% error.

Why Does Polyacrylamide Matrix Hamper Absolute Quantification? We see several lines of evidence that the reduced yield of tryptic peptides from in-gel digests resulted from the limited yield of in-gel digestion process, rather than from our inability to recover produced peptides from the gel matrix or to accurately determine their concentration. Peptide yield in the in-solution digest was almost complete; however, the recovery of peptides from in-gel digests was not. We then successively tested a number of factors that might be contributing to biased loss of digestion products. Although we observed that adding the internal standards into the mixture improved quantification, no indication of a major loss of peptides was apparent. At the same time, the only experi-

ment that improved the average peptide yield (although not to a satisfactory level) was digesting proteins by the higher concentration of trypsin under elevated temperature. We point out that, in these experiments, we were only interested in abundant peptides with C-terminal arginine moiety that are most suitable for reliable quantification.

Several factors might limit the efficiency of in-gel digestion. Slow diffusion of trypsin into a gel matrix or sterically hindered enzyme/substrate binding might contribute to diffusion control of the in-gel digestion kinetics. We previously observed that although the time of in-gel digestion could be reduced considerably without compromising the yield, the predigestion saturation of gel pieces with the enzyme solution could not.[22] The yield of digestion products was still increasing when dried gel pieces were incubated in a trypsin solution for more than 60 min, although gel pieces stopped absorbing water after 15 min. Slow penetration of trypsin into a gel matrix might not be surprising, since the estimated diameter of a cavity in 12% polyacrylamide gel is 4.4 nm, whereas the diameter of the trypsin globule is ~2.5 nm.[31] Taken together, these factors support the notion on diffusion control of the in-gel digestion process.

CONCLUSION AND PERSPECTIVES

A combination of tandem mass spectrometry and ^{18}O-labeled internal standards provided reliable absolute quantification of simple mixtures of proteins in solutions. Internal standards could be directly obtained by digesting a stock solution of the protein of interest with the known concentration in a buffer containing $H_2{}^{18}O$. Since many peptides can be quantified in parallel, the resulting estimate of the protein concentration is robust and would also be applicable to complex protein mixtures if combined with LC−MS/MS and powered by appropriate software. The method is also applicable to absolute quantification of gel-separated proteins, but its efficiency and accuracy are limited. Since the yield of individual peptides is biased, it is important to use several peptide peaks for quantification. Furthermore, such estimation could only be semiquantitative. A conservative estimation, based on a small selection of model proteins encompassed in this study, suggested a 50% error margin, even though the dispersion of determined concentrations of individual peptides is rather small. This, of course, does not rule out that for some proteins (for example, myoglobin) precise absolute quantification is possible. Our data point to intragel diffusion of the enzyme as a major factor that controls the in-gel digestion process. It is, therefore, unlikely that minor improvements in sample preparation might overcome those limitations. We concluded that using gels as a separation medium strongly affects the accuracy of the absolute quantification, and priority should be given to technologies that enable direct quantification in complex in-solution protein mixtures.[32]

We also note that encountered problems in absolute quantification of gel separated proteins might also affect their relative quantification.

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List of Abbreviations. nanoESI, nanoelectrospray ionization; MALDI, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SPE, solid-phase extraction; SD, standard deviation of the mean; and RSD, relative standard deviation.

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