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NOTES & TIPS

Evaluation of the Efficiency of In-Gel Digestion of Proteins by Peptide Isotopic Labeling and MALDI Mass Spectrometry

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Visualization of proteins separated by one-dimensional or two-dimensional polyacrylamide gel electrophoresis, in-gel digestion of excised protein bands (spots), followed by identification of proteins by mass spectrometry underpin many proteome characterization strategies (reviewed in (1–4)). A multitude of protocols for staining of polyacrylamide gels (reviewed in (5)) and of enzymatic in-gel digestion of proteins (reviewed in (1, 6)) at the low picomole–femtomole level has been reported.

A combination of protein visualization and in-gel digestion methods designed for proteomic application is usually evaluated according to two major criteria. First, the lowest limit of reliable visualization of protein spot (band) is determined (5, 7, 8). Second, the sequence coverage of MALDI peptide mass fingerprints acquired from in-gel digests of the visualized protein spots and the sensitivity of mass spectrometric detection are evaluated (8–10). However, the latter criteria are particularly difficult to apply. The intensity of peptide signals detected in complex mixtures by MALDI MS strongly depends on the employed sample handling and probe preparation routines. Furthermore, unavoidable micro-heterogeneity of MALDI probes and inevitable presence of the residual amount of dyes, salts, and detergents result in significant shot-to-shot variation of peptide and matrix signals, requires consistent tuning of the laser fluence (11) and, consequently, does not allow quantitative comparison of spectra acquired in the separate experiments.

Without direct measurement of the yield of peptide digestion products it is difficult to provide consistent evaluation of the efficiency of in-gel digestion protocols. Sequencing of proteins from silver-stained gels may serve as an example. Successful identification of silver stained proteins and high sequence coverage of MALDI peptide mass maps at the low femtomole level was reported (12), and almost identical peptide mass fingerprints were observed in the digests of silver and Coomassie stained protein bands (13). However, other authors reported substantially lower sequence coverage of peptide mass maps acquired from the digests of silver-stained proteins (8, 10, 14) that, however, could be improved by destaining of the bands prior to in-gel trypsinolysis (15, 16).

We therefore set out to develop a method for direct and quantitative evaluation of the efficiency of in-gel cleavage of proteins in order to outline a rational procedure for comparison of in-gel digestion efficiency. The yield of digestion products was determined by MALDI MS using ^{18}O-isotopically labeled peptides as the internal standards (17, 18). We further examined whether conventional methods of staining of polyacrylamide gels (Coomassie staining, silver staining, and zinc-imidazole staining) might affect the in-gel digestion efficiency.

Materials and Methods

Materials and reagents. All major chemicals were purchased from Sigma (Sigma Chemicals, St. Louis, MO) and were of analytical grade. $H_2{}^{18}O$ (Cambridge Isotopic Laboratories, MA) was purified by microdistillation as described (19).

Gel electrophoresis and visualization of protein bands. The aliquots containing 1 pmol of bovine serum albumin (Sigma Chemicals) in Laemmli buffer were loaded onto separate lanes of a one-dimensional polyacrylamide gel. Immediately after electrophoresis, the gel was cut. Separate parts of the gel slab each containing three lanes with the BSA standards were stained by various methods.

Coomassie staining and silver staining were performed as described (13). Zinc–imidazole staining (negative staining) was performed according to (20).

In a separate experiment the gel was stained with silver. Four BSA bands were excised from the gel and then destained with potassium ferricyanide and sodium thiosulfate as described (15). Stained and

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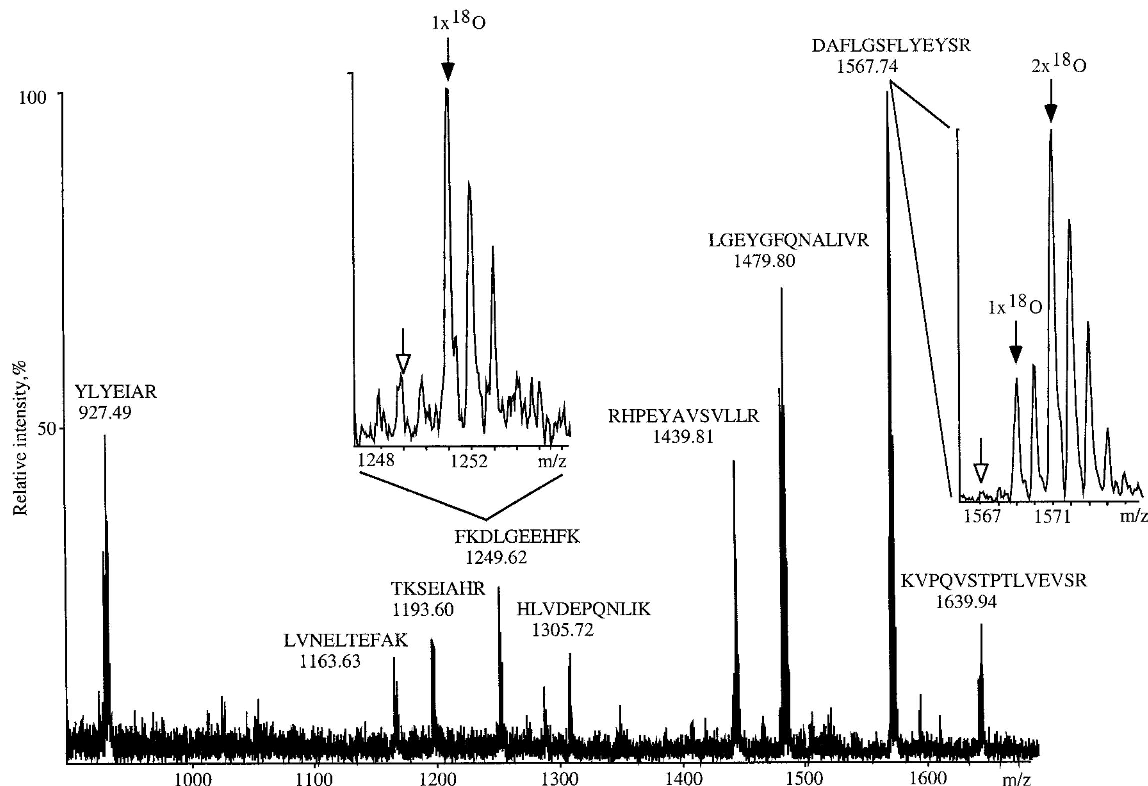

FIG. 1. A part of the spectrum of the tryptic digest of BSA in the buffer containing $H_2\,^{18}O$. Peaks in the spectrum are designated with corresponding peptide sequences and *m/z* calculated for the unlabeled monoisotopic ions. Blowouts demonstrate isotopic profiles typical for the peptide ions having arginine or lysine residues at their C-termini. The positions of the corresponding monoisotopic unlabeled ions are designated with unfilled arrows.

destained bands were further processed in parallel using conventional recipe.

In-gel digestion, preparation of sample probes and MALDI analysis. To prepare a standard mixture of ^{18}O-labeled peptides, a solution of 0.14 pmol/μL BSA in 25 mM ammonium bicarbonate buffer in $H_2{}^{18}O$ was digested overnight at 37°C; enzyme:substrate ratio 1:10 (w/w).

Protein bands (three for each method of staining) were in parallel in-gel digested with trypsin (unmodified, sequencing grade, Roche Diagnostics GmbH, Germany) as described (13, 19). Gel pieces were extracted with 5% formic acid and acetonitrile and the extracts were dried down in a vacuum concentrator. Tryptic peptides were redissolved in 10 μL of 10% formic acid. A 2-μL aliquot was withdrawn and mixed with 1 μL of an ^{18}O-labeled mixture of peptides (internal standard) prepared as described above. Four 0.5-μL aliquots of every mixed sample were analyzed in parallel by MALDI MS as described in (13, 21) on a modified REFLEX III mass spectrometer (Bruker Daltonics, Germany). The determined relative concentrations of peptides were averaged. The relative standard deviation of the concentrations in all series of measurements was better than 20%.

Results and Discussion

Quantification of peptides in in-gel tryptic digests. Upon digesting of a protein in the buffer, which contains $H_2{}^{18}O$, tryptic peptides incorporate one or two ^{18}O-atoms into their C-terminal carboxyl groups (22). Comparison of the peptide mass maps acquired from the digests of various standard proteins revealed that peptides, which contain arginine residue at their C-terminus incorporate two ^{18}O atoms (2× ^{18}O peptides) mostly, whereas peptides having C-terminal lysine residue incorporate one ^{18}O atom (1× ^{18}O peptides) (Fig. 1). Incubation of 2× ^{18}O peptides in 10% formic acid in $H_2{}^{16}O$ at room temperature resulted in a mixture of unlabeled, 1× ^{18}O and 2× ^{18}O forms. However, this process was slow and required several days before substantial alteration of the isotopic profile was detected (data not shown).

Isotopically labeled peptides produced by digesting of a protein in $H_2{}^{18}O$ could be employed as internal standards for quantitative measurements by MALDI MS. A standard mixture of ^{18}O-labeled peptides was prepared by digesting BSA with trypsin in solution in the buffer containing $H_2{}^{18}O$. Very similar profiles of tryptic peptides were detected in MALDI peptide

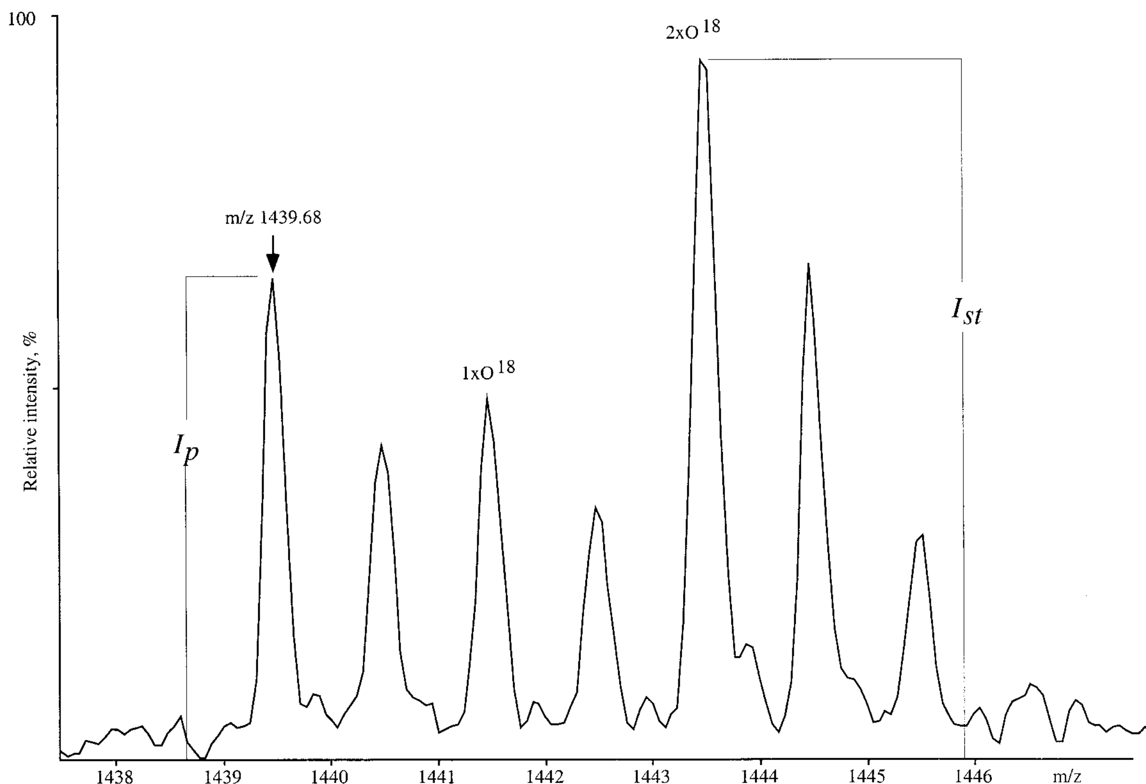

FIG. 2. Calculation of the relative concentration of peptides. A blowout of the isotopic cluster of the peptide peak with *m/z* 1439.93. The monoisotopic peak of the unlabeled peptide is designated with a filled arrow. The peak of the isotopicaly labeled peptide, which incorporated two ^{18}O atoms (2× ^{18}O) was used as an internal standard. The relative concentration of the unlabeled peptide (R_c) was calculated as $R_c = I_p/I_{st}$, where I_p stands for the intensity of the peptide peak and I_{st} stands for the intensity of the peak of the standard.

maps of in-solution digests and of in-gel digests, although the relative intensity of peptide peaks was altered. Equal volume of the mixture of ^{18}O-labeled peptides was spiked into the aliquots withdrawn from the experimental in-gel digests that were performed in $H_2{}^{16}O$ and the samples were analyzed by MALDI MS. Relative concentration of digestion products was calculated as a ratio of the intensity of the monoisotopic peak of the unlabeled peptide and the intensity of the monoisotopic peak of the corresponding 2× ^{18}O peptide standard (Fig. 2).

Linearity of the calibration curve was tested by analyzing the series of samples obtained by successive diluting of the aliquot withdrawn from the in-gel digest of 1 pmol of BSA. The relative concentrations calculated for various peptides were found linear over 1:5 dilution range and were affected by chemical noise at larger dilution ratios (data not shown).

The effect of gel staining on the recovery of tryptic peptides. This was examined by analyzing in-gel digests of the bands containing 1 pmol of BSA, which were stained with Coomassie, silver, and zinc-imidazole. A similar profile of tryptic peptides was detected

TABLE 1

Relative Concentration of Tryptic Peptides of BSA in In-Gel Digests of Bands Stained by Various Methods

	Relative concentration[a] (%)				
Staining method	*m/z* 927.49	*m/z* 1439.81	*m/z* 1479.80	*m/z* 1567.74	*m/z* 1639.94
Silver, with reduction and alkylation	100	100	100	100	100
Coomassie	105	136	74	95	121
Zn/Imidazole	117	61	74	79	100

[a] Relative concentrations of peptides were normalized to the concentrations in the digests of silver-stained bands.

TABLE 2

Relative Concentration of Peptides Recovered from Silver-Stained Gels

Sample preparation method	Relative concentration[a] (%)				
	m/z 927.49	m/z 1439.81	m/z 1479.80	m/z 1567.74	m/z 1639.94
With destaining, reduction and alkylation	100	100	100	100	100
With destaining only	95	110	93	97	98
With reduction and alkylation	115	104	102	83	94
Untreated[b]	<5	12	14	6	19

[a] Relative concentrations of peptides were normalized to the concentrations in the digests of destained, reduced and alkylated bands.
[b] Predigestion washing, destaining, reduction and alkylation were skipped.

in each of those samples (data not shown) and relative concentrations determined for five most intense peptide ions were compared. We observed slight variation of the relative concentration of individual peptides. However, no one method of staining provided significantly better recovery of peptides compared to other methods in the test (Table 1).

We further tested whether the recovery of peptides from silver stained gels could be improved by destaining of protein bands prior to in-gel digestion (15). To this end we compared the relative concentrations of peptides in the in-gel digests of the destained bands and of the bands treated according to the conventional protocol. We observed no significant increase in the number of detected peptide peaks as well as in the yield of peptides if destaining of bands was applied (Table 2). Similar conclusion was reached by Moertz *et al.* (12) on the basis of MALDI analysis of a large number of automatically processed samples. Reduction and alkylation steps did not influence the recovery of peptides, which do not contain cysteine residues and therefore for the purpose of protein identification those steps could, in principle, be omitted (23). Notably cysteine-containing peptides were missing if reduction and alkylation steps were skipped (Fig. 3). We therefore concluded that at the level of 1 pmol of protein starting material the method of protein visualization

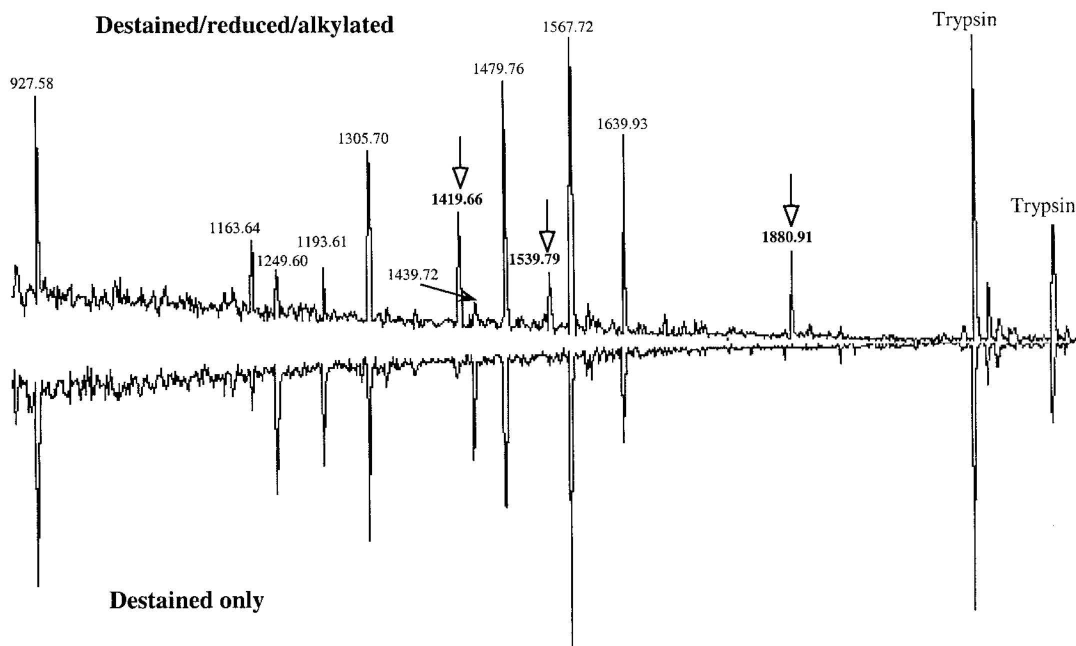

FIG. 3. Comparison of the peptide maps of the silver stained bands processed using destaining, reduction, and alkylation (the upper spectrum) and using only destaining (the lower spectrum). Peaks are designated with corresponding *m/z;* peptide sequences are presented in Fig. 1. Three intense peptide peaks (designated with unfilled arrows) having matching cysteine-containig peptides from BSA were additionally detected after reduction and alkylation. Corresponding peptide sequences are: *m/z* 1419.66 SLHTLFGDELCK; *m/z* 1539.79 LCVLHEKTPVSEK; *m/z* 1880.91 RPCFSALTPDETYVPK. C stands for cysteine-*S*-acetamide residue.

does not have any noticeable impact on the recovery of tryptic peptides.

We also observed that relative concentration of all peptides in the digests of silver-stained bands, which were directly treated with trypsin (i.e. washing steps as well as destaining, reduction and alkylation were omitted), was dramatically lower. Nevertheless, the number of detected peptide was always sufficient for unambiguous identification of BSA upon searching a database. We therefore speculate that the sequence coverage of MALDI peptide maps alone does not constitute an adequate measure of the digestion efficiency and should be complemented by direct quantification of peptide products.

Thus we have demonstrated that application of isotopically labeled peptide standards and MALDI MS enabled direct and quantitative evaluation of the efficiency of in-gel digestion. The method paves the way for further optimization of sample processing routines, thus improving sensitivity and throughput of the characterization of proteomes by mass spectrometry.

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Reutilization of Immunoblots after Chemiluminescent Detection

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Immunoblotting is widely utilized to evaluate the presence of antigens of interest in various biological samples, monitor antigen purification, assess epitope retention after antigen degradation, or assay for the presence of antibodies of a particular specificity in biological fluids [reviewed in Refs. (1–4)]. Under certain circumstances, e.g., if a blot suggests an unexpected difference in antigen expression between two samples or the antigens being analyzed are derived from a precious source, it can be important to sequentially probe the same blot for the presence of multiple antigens. The present study demonstrates that treatment with sodium azide after detection of bound HRP[1]-coupled secondary antibodies results in inhibition of the reporter group, thereby facilitating sequential probing of blots if reagents raised in multiple species are available.

A number of approaches have been previously proposed for the detection of multiple antigens on immu-

[1] Abbreviations used: HRP, horseradish peroxidase; PBS, calcium- and magnesium-free phosphate-buffered saline.

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