

Mass Spectrometry of Whole Proteins Eluted from Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis Gels

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In this report we describe a novel approach to the mass spectrometric analysis of whole proteins from gels. The strategy consists of three components: conventional SDS–PAGE gels, reversible negative staining procedures, and passive elution of proteins from gels followed by mass spectrometric analysis. Protein bands are excised from SDS–PAGE gels, destained, and extracted. For gel loadings ≥ 25 pmol of soluble protein, the proteins can be directly extracted into a solution consisting of formic acid/water/2-propanol. The recovered protein is suitable for matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization mass spectrometric analysis. For gel loadings < 25 pmol protein, the mass spectrometric response, using the direct extraction procedure, drops off sharply, an outcome that is attributed to protein recovery losses. To offset the protein losses, the extraction procedure is slightly modified by performing the passive extraction of the gel with a saturated MALDI matrix solution. During the extraction period, the matrix is allowed to crystallize, forming a suspension in solution. Protein that elutes from the gel has a chance to cocrystallize with the matrix that can be retrieved for MALDI–MS analysis. This method of “capturing” eluted protein into matrix crystals is sensitive to 1 pmol of recombinant mouse leptin protein (16 kDa) loaded onto SDS–PAGE gels and can be used for proteins as large as 70 kDa. Our strategy has particular application to the characterization of endogenous forms of mature proteins from SDS–PAGE gels. © 1997

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Mass spectrometry (MS) and electrophoresis are powerful methods that can provide microscale analyses of peptides and proteins. Each method exhibits unique strengths. For example, matrix-assisted laser desorp-

tion-ionization (MALDI)¹–MS (1) and electrospray ionization (ESI)–MS (2, 3) provide rapid, sensitive, and accurate molecular masses of peptides and proteins (4). Gel electrophoresis is a widely used approach for separating complex mixtures of proteins and obtaining estimates of protein molecular masses (5). The interfacing of these two methods is beginning to provide even more powerful analytical tools. The coupling of one- and two-dimensional polyacrylamide gel electrophoresis with MS is experiencing a surge of activity, particularly in the areas of protein identification using database-searching algorithms (6–8) and characterization of posttranslational modifications of proteins (reviewed in 9). These applications employ various types of gel separation and mass spectrometric techniques that depend on *in situ* chemical or proteolytic digestion of the protein of interest followed by elution of the digest peptide fragments for MS analysis.

Techniques are also being developed for the MS analysis of whole proteins from gels. The resulting molecular mass of a given intact protein provides key constraints for the elucidation of the primary structure of the mature protein. Any putative posttranslational modification and/or proteolytic processing of the protein must yield a mature protein product with a calculated mass that agrees with the measured mass. Retrieving large polypeptides and proteins from gels, however, is inherently more difficult than recovering small and moderately sized peptides originating from *in situ* digestion. In addition, with the use of SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), the presence of SDS detergent and protein visualization dye (e.g., Coomassie brilliant blue) severely compromises mass spectrometric re-

¹ Abbreviations used: 4HCCA, 4-hydroxy- α -cyano-cinnamic acid; ESI, electrospray ionization; FAPH, formic acid/acetonitrile/2-propanol/H₂O (50:25:15:10 by volume); FWI, formic acid/water/2-propanol (1:3:2 by volume); *m/z*, mass-to-charge ratio; MALDI, matrix-assisted laser desorption/ionization; TFA, trifluoroacetic acid.

sponse to proteins (S. L. Cohen, unpublished results; 10, 11). A variety of methods have been introduced to overcome these limitations and permit MS analysis of whole proteins from gels. One strategy uses electroblotting and electroelution techniques. For example, plasma desorption (12), uv-MALDI-MS (13), or ir-MALDI-MS (11) can be directly run on proteins that have been electroblotted onto polymeric membranes. Ultraviolet-MALDI-MS has also been applied to electroblotted proteins recovered from a dissolvable nitrocellulose membrane (14) and ESI-MS analysis to proteins passively eluted or electroeluted from SDS-PAGE gels (15-17). A more recent approach applies uv-MALDI-MS to the analysis of proteins directly from ultrathin polyacrylamide gels (10).

In this report we present an alternative strategy to mass spectrometrically analyzing whole proteins from gels. The method consists of three components: the use of conventional SDS-PAGE gels, reversible negative staining procedures, and passive elution of proteins from the gel followed by MALDI- or ESI-MS analysis. For high sensitivity applications, the method is restricted to MALDI-MS and involves the use of slow matrix crystallization (18). Our strategy offers the ability to analyze soluble proteins from gels down to the low picomole level (1-10 pmol), providing a powerful means for characterizing endogenous forms of mature proteins separated by SDS-PAGE.

EXPERIMENTAL

Proteins

Horse skeletal muscle myoglobin (17,568 Da; 95-100% pure; Sigma, St. Louis, MO) was accurately weighed on a microbalance (Cahn 4400, Orion Instruments, Boston, MA) and dissolved in aqueous 0.1% TFA (pH 2) (Pierce, Rockford, IL) to make a 50 μM protein solution. Recombinant mouse leptin was a gift from Dr. Jeffrey Friedman (Rockefeller University). The leptin consisted of wild-type residues 22-167 with an amino-terminal methionine (16,134 Da) (19). The presence of a single intramolecular disulfide bond in mouse leptin was determined by performing in-solution CNBr digestion as well as enzymatic proteolysis (20). The leptin concentration was 1 mg/ml (62 μM ; prepared in a standard phosphate-buffered saline solution, pH 7.4) and was determined spectrophotometrically using an extinction coefficient of 0.16 (mg-cm/ml)⁻¹ (personal communication, Ketan Gajiwala, Rockefeller University). The molecular mass marker set (Mark12; ~ 0.5 μg /protein band; Fig. 4) was from Novex (San Diego, CA). For qualitative studies (Fig. 6), a broad-range protein mass marker mixture (~ 0.2 μg /protein band) was purchased from New England BioLabs (Beverly, MA). Bovine transferrin and bovine carbonic anhydrase II were obtained from Sigma.

Preparation of Proteins for SDS-PAGE

Proteins were aliquoted into 0.65-ml microcentrifuge tubes and ammonium phosphate or acetate buffer was added to bring the volume to 10 μl . An equal amount (10 μl) of Tris-glycine sample buffer (2 \times concentrated) (Novex) was added to each tube. The sample buffer contained bromophenol blue to visualize the electrophoresis dye front. The protein/sample buffer solutions were vortexed and centrifuged prior to loading onto the gel.

SDS-PAGE

Gel electrophoresis was performed according to Laemmli (21). Gloves were worn at all times when handling the gels. Precast 14% Tris-glycine gels (1 mm thick, 10 wells) (Novex) were used throughout, although gels with other acrylamide concentrations, thicknesses, and numbers of wells can be used, depending on the desired molecular mass range, sample volumes, and electrophoresis equipment available. However, only Tris-glycine gels are appropriate for the present application because the destaining process is based on the Tris buffer system (22). Electrophoresis was carried out on an Xcell II Mini-Cell device (Novex) at room temperature. The electrophoresis running buffer was prepared from a 10 \times Tris-glycine-SDS solution (Novex). Following assembly of the Mini-Cell with a fresh gel and addition of the running buffer, the protein/sample-running buffer solutions (20 μl ; see above) were loaded into the sample wells with the aid of gel-loading tips (Novex). Electrophoresis was carried out at 120 V and an initial current of ~ 32 mA using a PowerPac 1000 power supply (Bio-Rad; Hercules, CA) and was terminated when the dye front reached close to the bottom of the gel. Typical run times were 1.5-2 h.

Gel Staining

Immediately following electrophoresis, the gels can be rinsed in water, although we recommend against rinsing when handling low-molecular-mass proteins (<20 kDa) that are at low picomole gel-loading amounts. Protein bands were visualized by soaking the entire gel in 45 μl copper-staining solution (supplied 10 \times from Bio-Rad) for 5 min with gentle rocking (22). Alternatively, zinc-staining (23) or imidazole-zinc-staining (24) protocols can be used. After staining, the gels were thoroughly rinsed in deionized water. Because copper and zinc staining are negative stains, protein bands appear translucent and are best viewed against a black background. Bands with as little as 17 ng (~ 1 pmol) of myoglobin or recombinant leptin were visualized by copper staining.

Gel Excising and Destaining (Fig. 1)

Destaining was performed on individual gel slices containing a single protein band as depicted in Fig. 1.

Figure 1, step 1. Protein bands were excised from the stained gel with a clean, sharp straight-edge razor (Fig. 1). The gel slices were excised as close as possible to the boundaries of the protein band. The resulting blue-tinted gel slices typically had the dimensions 1–1.5 mm × 6–7 mm and were best handled with flat-end tweezers. Protein extraction should be performed immediately after destaining. If a delay is anticipated, it is best to leave the gel slices stained and stored moist (but not soaking) at 4°C in a sealed microtube until needed.

Step 2. The destaining process completely removes the visualization stain as well as the SDS detergent (22). Each gel slice was treated in a separate 1.5-ml microcentrifuge tube using standard copper-destaining protocols (Bio-Rad). Zinc-stained gels can also be destained using the same protocols. Destaining was performed by a three-step soaking process using a copper Tris–glycine destain solution (Bio-Rad). The first soaking of the gel is carried out in a solution consisting of 100 μ l destain buffer and 900 μ l water. The tube is sealed and vortexed (Tomy MT-360 36-sample microtube mixer; Tomy Tech, Palo Alto, CA) for 5 min at room temperature. During the first destaining period the solution turns pale translucent blue (for copper-stained gels) and may become slightly foamy. The gel will retain a faint blue tint. The second soaking is performed in fresh solution of 100 μ l destain and 900 μ l water with 10 min of vortexing. At the end of the second soaking, the destain solution and gel will be nearly colorless. The final soaking is performed in a solution of 50 μ l destain and 950 μ l water with 5 min of vortexing. At the end of the third soaking, the destain solution as well as the gel are clear. The destained gel pieces are rinsed in deionized water and are ready for extraction.

Step 3. Prior to protein extraction, the gels are crushed. Before crushing, the destained gel slices are gently blotted free of any excess water clinging to the gel with a lint-free tissue, a step that facilitates crushing of the gel. With the aid of tweezers, the gel slices are placed into a 0.65-ml polypropylene microcentrifuge tube (PGC Scientifics, Gaithersburg, MD). The microtube should be of high quality, free of plasticizers and contaminants that can compromise high sensitivity MS measurements. From our experience, we avoid the use of colored microtubes. The gel slice is manually crushed at the bottom of the microtube for a few seconds with a sharp-pointed dental tool. Mechanical homogenization of the gel can be used instead of the manual crushing, but we recommend against it since the resulting small gel pieces tend to clog pipettor tips.

At this point, either of two methods described below (Extraction Methods A or B) can be used to extract the protein.

Extraction Method A (Fig. 1)

This method of extraction elutes protein directly into an aqueous solution and is best suited to gel loadings exceeding 25 pmol. Solution compositions that we have examined for protein extraction include formic acid/water/2-propanol (1:3:2 v/v/v) (FWI), formic acid/water (1:5 v/v), water/2-propanol (2:1 v/v), and pure water. Optimal extraction efficiency occurred with the FWI combination, whereas no extraction was observed with pure water. (The relative extraction efficiencies are based on a qualitative assessment of the MALDI–MS signal-to-noise ratios). Other acids and water-miscible organic solvents (e.g., 0.1% TFA and acetonitrile) can be used for extraction.

Figure 1, step 4a. Sufficient extraction solution (~30–40 μ l) is added to the crushed gel to completely cover the gel pieces.

Step 5a. The tube is closed and vigorously shaken or vortexed at room temperature from 4 to 8 h. Although not essential for protein elution, shaking/vortexing facilitates protein extraction. Generally, the greater the protein loading or the smaller the protein, the shorter the vortexing time required for extraction.

Step 6a. After the vortexing period, the microtube is centrifuged and the supernatant is retrieved with a 10- μ l micropipettor, avoiding taking up any of the gel pieces. The crushed gel is washed once with an equal volume of fresh extraction solution and the wash is combined with the supernatant.

Steps 7a and 8a. If the protein is to be analyzed by MALDI–MS, the extraction solution is lyophilized and a MALDI matrix solution (2 μ l for Fig. 2) is added to redissolve and retrieve the protein for MS analysis using the dried-drop method of matrix crystallization (see below). The matrix solution is FWI saturated with 4-hydroxy- α -cyano-cinnamic acid (4HCCA) (25). If the protein is to be analyzed by ESI–MS, the extraction solution is injected into a protein-desalting cartridge (Michrom BioResources, Inc., Auburn, CA) that is mounted to the ESI capillary inlet. The loaded cartridge is flushed several times with a 2% acetic acid wash solution. Following the washing, the protein is eluted from the cartridge into the electrospray source with a solution consisting of 70% acetonitrile/2.5% acetic acid at 6 μ l/min. ESI–MS was performed on a TSQ-700 triple quadrupole (Finnigan Corp., San Jose, CA) using standard ESI conditions for analyzing proteins (26).

Extraction Method B (Fig. 1)

This method of extraction elutes protein from gels directly into a MALDI–MS matrix solution and is best

suiting to gel loadings below 25 pmol of protein or for proteins that fail to passively elute using Extraction Method A (described above). In Method B the matrix is allowed to undergo slow crystallization (18) during the protein extraction period. The matrix used throughout was 4HCCA, although sinapinic acid can also be used. The moderately low solubility of these cinnamic acid derivatives in the matrix solution is an important characteristic for efficient slow crystallization (18). The most frequently used matrix solution in our experiments consisted of a saturated solution of 4HCCA in FWI. Other solvent systems were also examined (e.g., 0.1% TFA:acetonitrile, 2:1 v/v) (25). Gel extraction is as follows.

Figure 1, step 4b. After crushing the destained gel (see above), enough matrix solution (~ 30 – $40 \mu\text{l}$) is added to cover the gel pieces.

Step 5b. The tube is closed and vigorously shaken or vortexed at room temperature for 1–2 h.

Step 6b. Matrix crystallization is induced by leaving open the top of the microtube (0.5–1 h) with vortexing. The matrix solution will become slightly cloudy with a suspension of 4HCCA crystals. An aliquot ($1 \mu\text{l}$) of the milky supernatant can be immediately retrieved and deposited onto the MALDI probe for MS analysis while the remaining sample is closed and left vortexing for additional extraction. Avoid taking up any gel pieces while pipetting and avoid crushing the matrix crystals once they are on the probe.

Steps 7b and 8b. If the MALDI–MS response from the “immediately retrieved” crystals is weak, the signal should improve from crystals that have undergone a longer period (~ 4 – 24 h) of vortexing in the gel/matrix suspension. During extended vortexing periods, the matrix crystals precipitate to the bottom and/or cling to the sides of the microtube and can be retrieved with a micropipettor for MALDI–MS analysis. Also, during the vortexing period volume losses of the solution due to evaporation and aliquot sampling may occur. To offset the volume losses, small amounts (5– $10 \mu\text{l}$) of matrix-free extraction solution are added to keep the gel pieces completely submerged in solution. For the results given in this paper vortexing times ranged from 6 h (Fig. 5) to 12–19 h (Fig. 6).

MALDI–MS

The dried-drop method of matrix crystallization (27) (see spectra in Figs. 2 and 3a) involves depositing an aliquot (0.5– $1 \mu\text{l}$) of sample–matrix solution onto an aluminum 10-sample MALDI probe. The droplet of solution is allowed to air dry (several minutes) at room temperature, resulting in a uniform layer of fine granular matrix crystals. Slow crystallization (18) (see spectrum in Fig. 3b) was performed on $30 \mu\text{l}$ of sample–

matrix solution in a small microcentrifuge tube. The tube was left open at room temperature for 3 h, after which time small matrix crystals appear in the solution. Vortexing hastens crystallization. The supernatant was removed and the remaining matrix crystals were washed twice with cold water, retrieved in 1– $2 \mu\text{l}$ of water, and deposited onto the MALDI probe with a micropipet tip. The solution was allowed to air dry, leaving matrix crystals firmly adhering to the probe.

All MALDI (uv-MALDI) spectra were obtained on a linear time-of-flight mass spectrometer equipped with a 10-position multiple-sample probe. A full description of the instrument has been given earlier (28–30) and complete operating conditions can be found elsewhere (25) with the exception of the digital oscilloscope (Model 9350M, LeCroy Corp., Chestnut Ridge, NY) and data collection. For this report, spectra were collected on a Pentium PC using the programs “Acquire” and “M/Z,” the latter of which is publicly available on the World Wide Web (URL <http://mcphar04.med.nyu.edu/software/contents.htm>). The time-of-flight data were either externally calibrated or mass converted using internal calibrants.

RESULTS

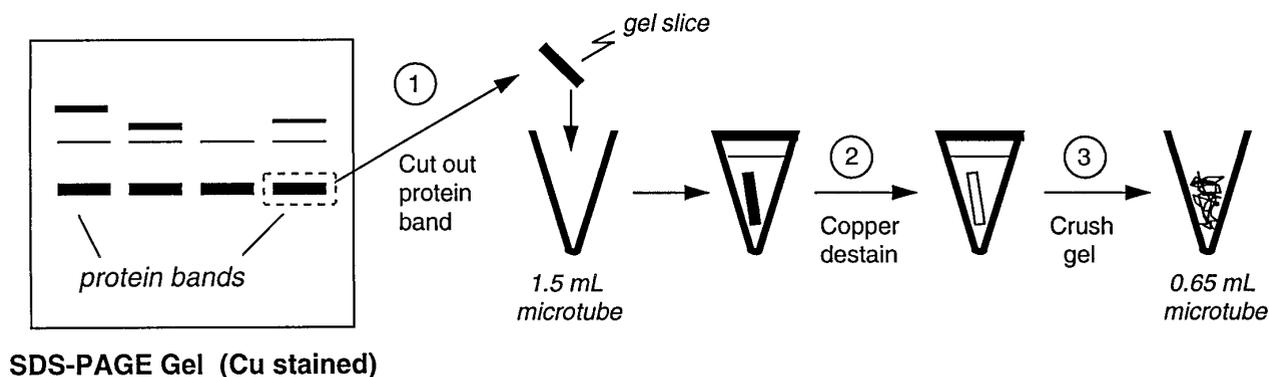
Eluting Whole Proteins from SDS–PAGE Gels

Our strategy for eluting whole proteins from SDS–PAGE gels is presented in Fig. 1. Full details of the procedures are given under the Experimental section. The approach starts with an SDS–PAGE gel on which proteins have been resolved. Protein bands are visualized with a reversible negative stain such as copper (22) or zinc (23, 24) staining. The bands are excised from the gel and destained. The destaining procedure completely removes the stain and SDS detergent. The gel is then crushed and the protein extracted by one of the protocols we call Extraction Methods A and B. We have found that for gel loadings ≥ 25 pmol of protein, the protein can be passively eluted directly into an extraction solution (Extraction Method A). For gel loadings < 25 pmol of protein, the inclusion of a MALDI matrix is important to ensure high sensitivity (Extraction Method B).

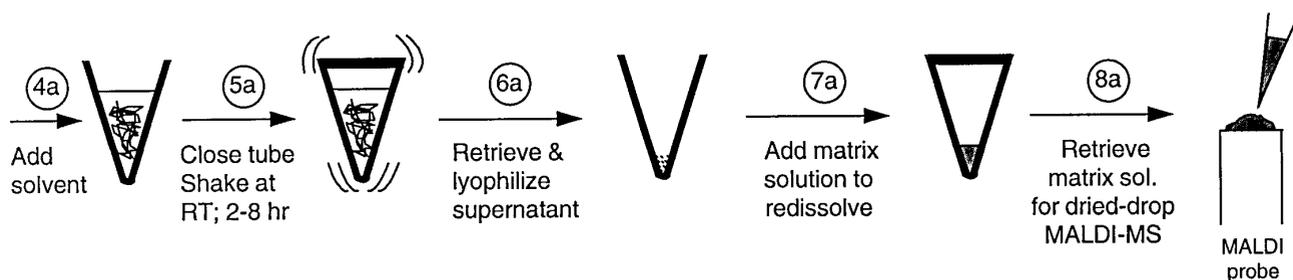
Extraction Method A: Elution of Whole Proteins from Gels for MS Analysis

This method involves a passive extraction of the protein into solution (Fig. 1 and see Experimental). Following extraction, the extraction solution is retrieved from the gel pieces and lyophilized. Saturated matrix solution is added to the lyophilized protein and MALDI–MS is run on the matrix crystals prepared by the dried-drop method. Figure 2 shows MALDI mass spectra of equine myoglobin and recombinant mouse

Gel Excision and Destaining



Gel Extraction Method A



Gel Extraction Method B

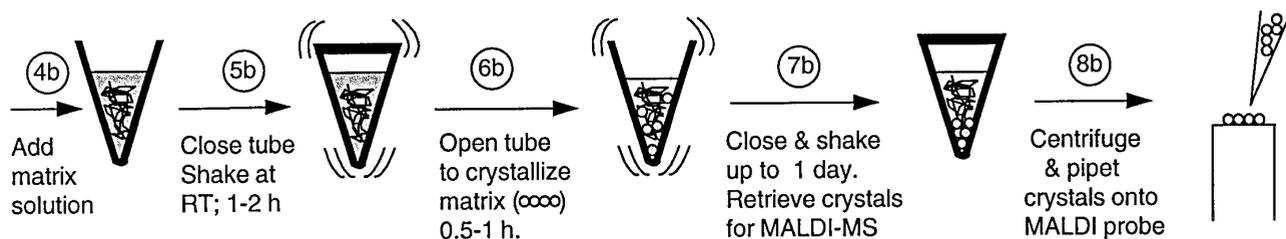


FIG. 1. Eluting whole proteins from copper-stained SDS-PAGE gels for mass spectrometric analysis. The strategy is composed of two parts. The first part consists of gel excising, destaining, and crushing (top row). The second part consists of gel extraction, involving either passive elution of the gel using an acidic solution (middle row, Gel Extraction Method A) or passive elution combined with a slow matrix crystallization using a MALDI matrix solution (bottom row, Gel Extraction Method B). See text for a full description.

leptin extracted by Method A. The spectra in Figs. 2a and 2b, obtained from the extraction of gels containing 25 pmol of the two proteins, show excellent signal-to-noise ratios, suggesting favorable protein extraction by Method A. However, because accurate quantitation by MALDI-MS is difficult, we did not estimate the recovery yields. MALDI mass spectra were also obtained for 50-pmol gel loadings of bovine carbonic anhydrase II

(29 kDa) and bovine transferrin (78 kDa) using Extraction Method A (data not shown). As an alternative to the MALDI-MS technique, ESI-MS can be used to analyze the extracted protein. For example, good quality ESI mass spectra from the extraction of 50-pmol gel loadings of myoglobin and leptin were obtained (data not shown).

The utility of Extraction Method A, however, is lim-

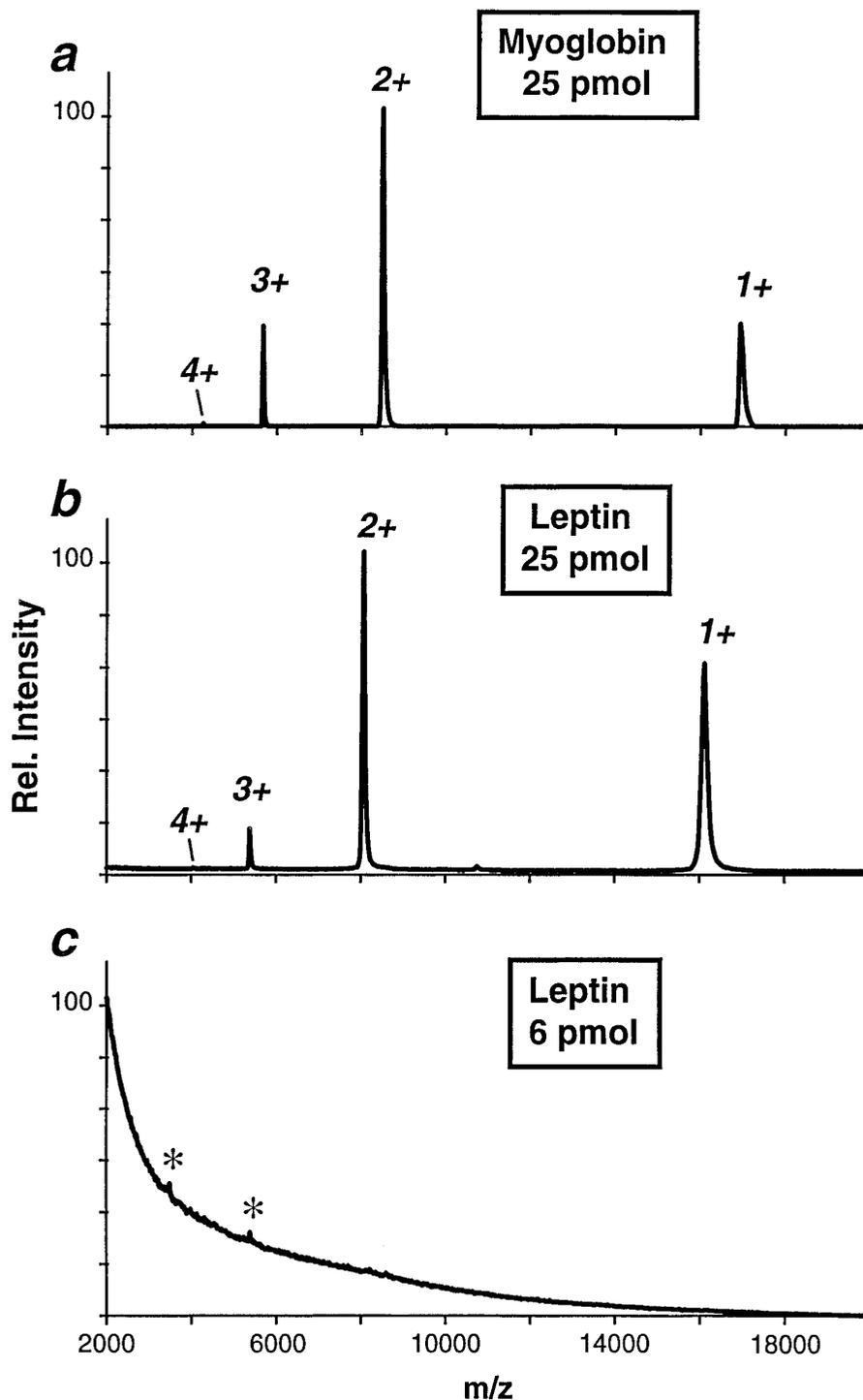


FIG. 2. MALDI-MS of proteins eluted from copper-stained SDS-PAGE gels using Extraction Method A (see text). The extraction solution was formic acid/water/2-propanol (1:3:2 v/v/v). The matrix solution had the same composition but was saturated with 4HCCA. The dried-drop method was used to crystallize the matrix for MALDI-MS analysis. MALDI spectra of three gel loadings are shown: (a) 25 pmol equine myoglobin; and (b) 25 pmol and (c) 6 pmol of recombinant mouse leptin (calc. mass 16,134 Da). The singly (1+) and multiply (2+ to 4+) protonated peaks arising from the MALDI mass spectrometric process are labeled. Since the MALDI matrix conditions are strongly acidic (i.e., denaturing), the peaks in (a) correspond to apomyoglobin (calc. mass 16,951 Da). The small peaks labeled with an asterisk in (c) are low-level impurities.

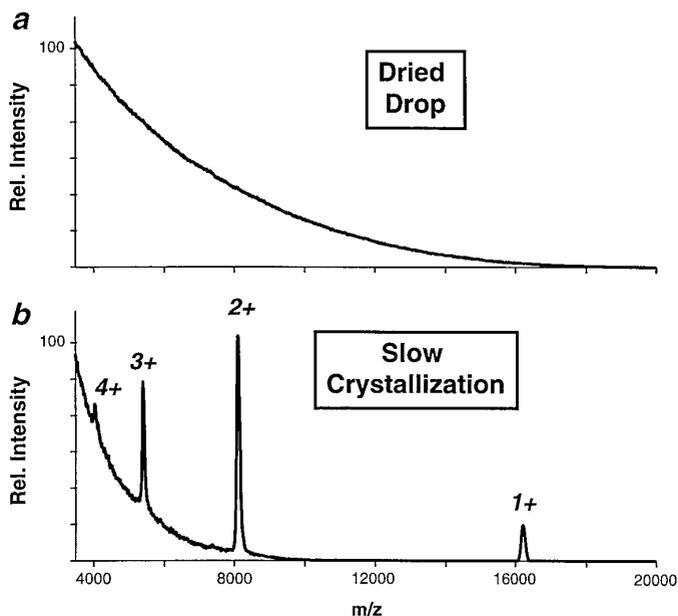


FIG. 3. Slow crystallization of the MALDI matrix enhances the MALDI-MS sensitivity of proteins. Mass spectra of recombinant mouse leptin (calc. mass 16,134 Da) using (a) the conventional dried-drop method (25) and (b) the slow crystallization method (16). The matrix solution was formic acid/water/2-propanol (1:3:2 v/v/v) saturated with 4HCCA. Protein concentration in the matrix solution was $0.01 \mu\text{M}$. The spectra labels are described in the legend to Fig. 2.

ited to gel loadings ≥ 25 pmol. As observed in Fig. 2c, the MALDI-MS signal significantly drops for gel loadings approaching the 6-pmol level of leptin. A similar fall-off in sensitivity is also observed with myoglobin (data not shown). We attribute the fall-off in signal to a reduction in protein extraction recovery yields. To offset the poor recovery yields from gels with low picomole loadings, a modification in the passive extraction method was introduced. The modification makes use of MALDI-MS analysis along with slow matrix crystallization as described below.

Slow Crystallization of the MALDI Matrix Greatly Enhances MALDI-MS Sensitivity

MALDI-MS depends on growing analyte-doped matrix crystals. Several methods of matrix crystallization are available (31), the most widely used of which is the dried-drop method (27). In the dried-drop method, proteins are dissolved in a saturated MALDI matrix solution. Ideally, the protein concentration in the matrix solution should be $>0.1 \mu\text{M}$ to ensure a good MALDI-MS response. Below $0.1 \mu\text{M}$ protein in the matrix solution, we observe a nonlinear drop-off in the MALDI-MS response (Fig. 3a). Figure 3a shows the MALDI mass spectrum of recombinant mouse leptin using the dried-drop method ($1 \mu\text{l}$ of matrix solution

containing $0.01 \mu\text{M}$ protein). No signal from the protein is observed. In striking contrast, when slow crystallization (18) is used to produce matrix crystals ($30 \mu\text{l}$ of matrix solution containing $0.01 \mu\text{M}$ protein), we observe a moderately strong MS response (Fig. 3b). A similar pattern of behavior is seen from a matrix solution containing $0.01 \mu\text{M}$ equine myoglobin (data not shown). Slow matrix crystallization thus provides a convenient means to concentrate protein from the solution into the matrix, a property that can be exploited to enhance MALDI-MS sensitivity.

Extraction Method B: Elution of Whole Proteins from Gels by a Matrix Solution for MALDI-MS Analysis

To achieve high sensitivity analysis of proteins originating from gels, we combined passive gel elution (Extraction Method A) with slow matrix crystallization. The result is a new approach to mass spectrometrically analyzing proteins from gels (Fig. 1, Extraction Method B). In Method B the eluting solvent is a MALDI matrix solution. The matrix solution permits passive extraction of the gel along with a nearly simultaneous slow crystallization of the matrix. Under vortexing conditions, the matrix crystals remain finely dispersed; protein that is eluted from the gel has a chance of cocrystallizing with the matrix suspension. We applied this approach of analysis to myoglobin and recombinant mouse leptin. Figure 4 shows a copper-stained SDS-PAGE gel of a titration of the two proteins whose gel loadings range from 25 to 1 pmol (~ 425 –17 ng). Since leptin shows an anomalously high electrophoretic mobility compared to myoglobin, the two proteins are well resolved in the gel. Figure 5 depicts the MALDI mass spectra of mouse leptin eluted from a copper-stained gel using Extraction Method B. The spectra demonstrate the effects of decreasing gel loadings of leptin, from 25 pmol (Fig. 5a) to 1 pmol (Fig. 5c). Extrac-

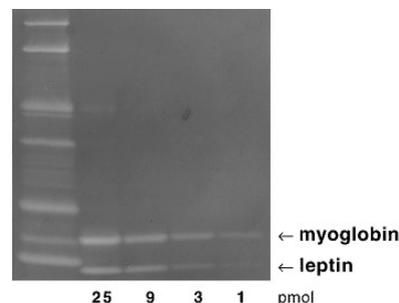


FIG. 4. SDS-PAGE of myoglobin and recombinant mouse leptin. Copper-staining methods (22) were used to visualize the gel. The left-most lane consists of molecular mass markers. The other four lanes show a titration of a mixture of myoglobin (apomyoglobin, calc. mass 16,951 Da) and recombinant mouse leptin (calc. mass 16,134 Da). The gel loadings are 25, 9, 3, and 1 pmol/protein. Two well-resolved bands are observed: the upper band is apomyoglobin and the lower band is leptin.

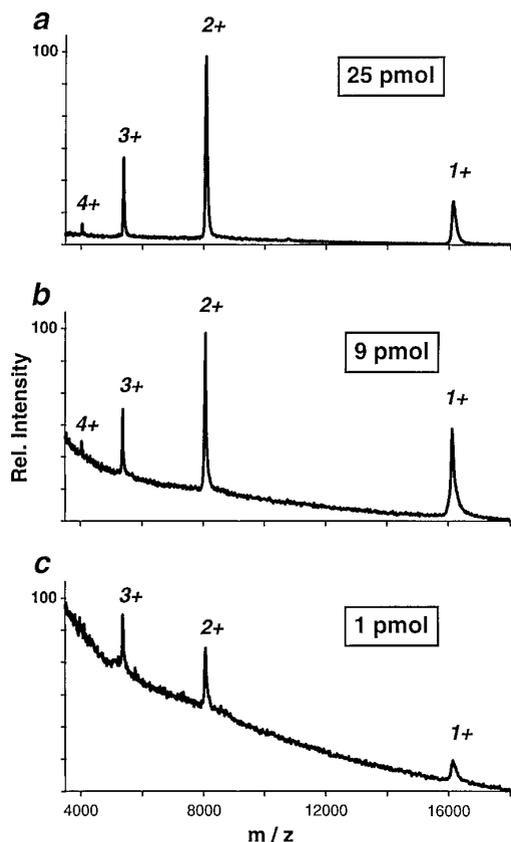


FIG. 5. MALDI-MS of recombinant mouse leptin (calc. mass 16,134 Da) eluted from a copper-stained SDS-PAGE gel using Extraction Method B. The MALDI matrix solution used to extract the gel slices was formic acid/water/2-propanol (1:3:2 v/v/v) saturated with 4HCCA (see Experimental). The gel loadings represent (a) 25 pmol, (b) 9 pmol, and (c) 1 pmol of recombinant mouse leptin. The spectra labels are described in the legend to Fig. 2.

tion Method B maintains good sensitivity even at 1 pmol gel loading of leptin, a level at which direct elution (Extraction Method A) completely failed to give any MALDI-MS signal for the protein (Fig. 2c). Similar MALDI-MS results were obtained for myoglobin (data not shown).

The MALDI mass spectra of a selection of protein mass markers obtained using Extraction Method B are shown in Fig. 6. Based on the marker set manufacturer's specification, the gel loadings were calculated at 200 ng for each protein. This represents 14 pmol of lysozyme (14.3 kDa; Fig. 6a), 7 pmol of triosephosphate isomerase (26.6 kDa; Fig. 6b), and 3 pmol of bovine serum albumin (66.4 kDa; Fig. 6c). Figure 6a exhibits an additional set of peaks whose mass correlates to the mass marker aprotinin (6.5 kDa), indicating that aprotinin comigrated with lysozyme on the SDS-PAGE gel.

Mass Accuracy

The MALDI mass spectra shown in Figs. 2, 3, 5, and 6 are externally calibrated. External calibration of pro-

teins using the dried-drop method normally yields mass accuracies in our simple linear time-of-flight mass spectrometer no better than 0.1%. With slow crystallization, other factors contribute to further reduce mass accuracy. For example, slow crystallization tends to produce larger and more heterogeneous matrix crystals than crystals formed by the dried-drop method. Also, a complication of using Extraction Method B is the inadvertent retrieval of microscopic gel pieces along with matrix crystals. Large matrix crystals and the inclusion of gel pieces on the MALDI probe may act to distort the electric field in the ion source. These effects lead to peak broadening and centroid shifts that ultimately reduce the mass accuracy. The use of internal calibrants leads to substantial improvements in mass accuracy. Using Extraction Method A, a protein of known molecular mass can easily be included in the

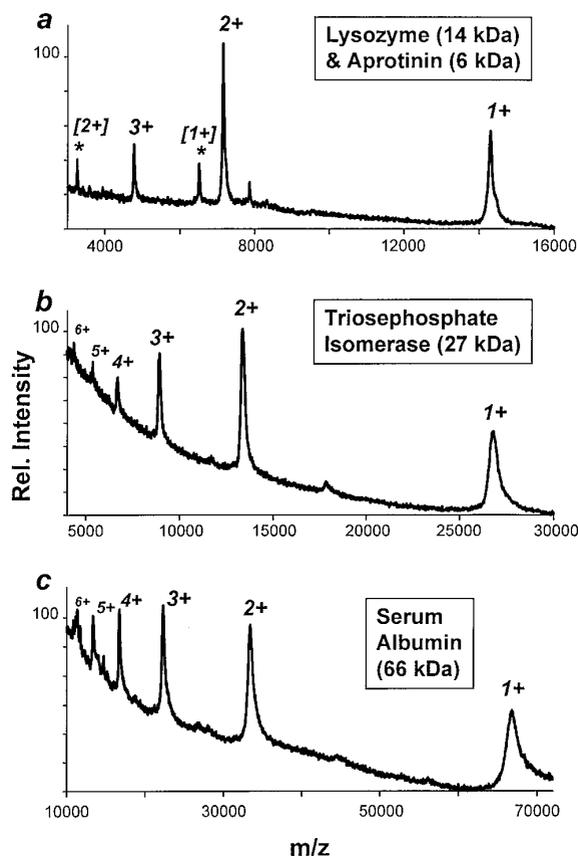


FIG. 6. MALDI-MS of protein molecular mass markers eluted from copper-stained SDS-PAGE gels using Extraction Method B. The MALDI matrix solution used to extract the gel slices was formic acid/water/2-propanol (1:3:2 v/v/v) saturated with 4HCCA (see Experimental). The markers are (a) chicken egg white lysozyme (calc. mass 14,313 Da); (b) triosephosphate isomerase (26,625 Da); and (c) bovine serum albumin (66,409 Da). The spectrum in (a) also shows peaks (labeled with asterisks) that indicate comigration and extraction of the marker protein aprotinin (6517 Da) along with the lysozyme. The spectra labels are described in the legend to Fig. 2.

matrix solution prior to dried-drop crystallization (Fig. 1, step 7a). Using Extraction Method B, the protein calibrant must be included before slow crystallization of the matrix (Fig. 1, step 4b). The amount of calibrant to be added depends on the level of sensitivity and must be established on a trial-by-error basis.

An example of mass accuracy obtained using Extraction Method B with an internal calibrant is as follows. A destained gel slice containing 9 pmol of recombinant mouse leptin was eluted by Method B. Equine myoglobin (1 pmol) was added to the matrix solution (at step 4b, Fig. 1) and protein elution/slow matrix crystallization was allowed to proceed. The resulting MALDI mass spectra of matrix crystals retrieved from the extracted gel showed a signal-to-noise similar to the spectrum in Fig. 5b (data not shown). Using myoglobin as the internal calibrant, the mass of the recombinant mouse leptin (calculated molecular mass 16,134 Da) was measured to be $16,133 \pm 6$ Da. The measured value is an average of three repeat MALDI spectra of the singly and doubly charged peaks of leptin and the estimate of error is the standard deviation.

Another strategy to improve mass accuracies of proteins is to use ESI-MS. For example, mass accuracies of $\sim 0.01\%$ were obtained for 50-pmol gel loadings of recombinant leptin eluted by Method A (data not shown). However, ESI-MS can only be used in conjunction with Extraction Method A and for gel loadings ≥ 25 pmol because ESI is intolerant to the MALDI matrix that is present in Extraction Method B.

As a note on reproducibility, we have performed an extensive number of gel elutions using Extraction Methods A and B over a 4-month period and have found relatively good MS reproducibility. As an example, separate extractions of freshly prepared gel slices, containing 3- to 25-pmol loadings of recombinant mouse leptin, were performed at different times over a 4-month period. The resulting MALDI mass spectra of the extracted gels using Method B yielded similar signal-to-noise ratios consistent with those shown in Fig. 6.

DISCUSSION

Our approach of mass spectrometrically analyzing whole proteins from SDS-PAGE gels depends on passive elution methods. The most efficient and frequently used passive gel elution employs buffer systems that normally contain SDS detergent (32). However, the presence of ionic detergents, such as SDS, usually severely compromises MS response by either ESI or MALDI techniques (S. L. Cohen, unpublished results; 30, 33). Thus, it is imperative to eliminate the detergent. When microgram quantities of protein are involved, detergent removal is normally carried out by protein precipitation with organic solvents such as cold acetone (32) or by ion-pairing methods (34). However,

the precipitation procedures are lengthy and usually become inefficient for sub-microgram quantities of protein.

Part of our aim in this study was to develop a means to mass spectrometrically analyze sub-microgram (low picomole) amounts of whole proteins loaded onto conventional gels with minimal sample handling. Reverse staining procedures using transition metal salts provided the desirable qualities to achieve our goal (35). Copper staining of SDS-PAGE gels, for example, shows moderately high sensitivities (~ 10 ng protein) (22, 36). More importantly, the copper-destaining protocols are simple and result in complete removal of the copper stain and SDS detergent with minimal loss of protein, leaving the gel prepared for passive elution.

We found that passive elution of destained gels using the solvent mixture FWI (Extraction Method A) was sufficient to extract soluble proteins for MALDI- or ESI-MS analysis for protein loadings ≥ 25 pmol (~ 0.5 μ g of myoglobin or leptin; Figs. 2a and 2b). We selected FWI because formic acid together with 2-propanol has excellent protein-solubilizing properties (37). Extraction solutions of differing compositions have been used by others, including the mixture formic acid/acetonitrile/2-propanol/water (FAPH) (50:25:15:10, by volume) (38). FAPH was noted particularly for its ability to elute hydrophobic proteins, such as membrane proteins, with high efficiency. Typical recoveries of hydrophilic (soluble) or hydrophobic (membrane) proteins (5- to 10- μ g loadings on SDS-PAGE gels) using passive elution by FAPH were reported to exceed 90% using radioactively labeled polypeptides (38). We examined FAPH as an eluant for Extraction Method A for gels containing 25 and 6 pmol of either recombinant mouse leptin or myoglobin and found MALDI-MS signals comparable to those observed by eluting with FWI (i.e., Fig. 2).

Below the 25-pmol gel loading of soluble protein, Extraction Method A showed a substantial nonlinear decline in the mass spectrometric response (e.g., Fig. 2c). For Fig. 2c, we attribute the disappearance of the MALDI-MS signal to insignificant protein recovery from the gel at the low picomole levels of gel loading. The recovery losses are probably due to irreversible adsorption of eluted protein onto the microtube and dispenser tip surfaces, loss mechanisms known to dominate for low picomole amounts of protein (personal communication, Salvatore Sechi, Rockefeller University). To counter protein losses at low picomole levels, we combined the passive elution procedure with a second process—slow crystallization of the MALDI matrix (18).

Beavis initially described slow crystallization as a powerful strategy to grow analyte-doped matrix crystals in the presence of high levels of involatile additives, conditions that normally preclude matrix crystallization using the dried-drop method (18). We have ob-

served an additional benefit of slow crystallization—a significant improvement in the MALDI response of proteins present in the matrix solution in low amounts over that observed using the dried-drop method (compare spectra in Fig. 3). Thus, slow crystallization is a powerful means of concentrating protein into matrix crystals, providing the rationale behind Extraction Method B. In Method B, protein that elutes from the gel has a high chance of being “captured” by the matrix crystals present in the extraction solution (e.g., Figs. 5 and 6).

The present method of eluting picomole amounts of proteins from gels for MS analysis can be applied to the study of endogenous forms of proteins. This application is particularly relevant for eukaryotic proteins that normally undergo extensive *in vivo* processing events following protein translation (e.g., proteolytic cleavage, disulfide formation, phosphorylation, and glycosylation). Characterizing posttranslational modifications is routinely performed by evaluating proteolytic digests by MS peptide mapping. However, peptide coverage of protein digestions by MS is frequently incomplete, a situation that can lead to ambiguous results. Knowledge of the accurate molecular mass of the intact protein along with information from digestions and peptide mapping can lead to more definitive results. This approach was used in a recent characterization of endogenous human leptin (39). Using Extraction Method B on immunopurified leptin from blood serum drawn from obese human donors, the molecular mass of the intact native leptin was determined. In this experiment recombinant human leptin was used as a control and myoglobin was used as the internal calibrant. In-gel digests with trypsin and cyanogen bromide followed by MALDI-MS peptide mapping were also performed on the endogenous and recombinant proteins. The results of this study unambiguously showed that endogenous human leptin had the wild-type sequence with the predicted posttranslational processing (39). The characterization was completed in less than 2 days. We note that in order to hasten the analysis of a large number of protein gel bands, the gel destaining/extraction procedures can be scaled up by processing the samples in a parallel manner.

There are several possible shortcomings with our method of eluting picomole amounts of proteins from gels. Hydrophobic proteins or very large proteins may resist passive elution. However, the use of different solvent systems (e.g., FAPH, see Results) (38) or the inclusion of certain nonionic detergents (e.g., *n*-octylglucoside) may assist in the elution. Also, as discussed previously, obtaining sufficient mass accuracies using Extraction Method B can be a significant concern because of the heterogeneous matrix layer. Careful selection of internal calibrants and repetitive measurements usually provide mass accuracies $\leq 0.1\%$. It is

likely that the newly developed SDS pulsed ion extraction methods for the MALDI ion source (40–42) will provide a means to “correct” the deleterious effects of the heterogeneous matrix layer (43).

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