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Mass Spectrometric Sequencing of Proteins from Silver-Stained Polyacrylamide Gels

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Proteins from silver-stained gels can be digested enzymatically and the resulting peptides analyzed and sequenced by mass spectrometry. Standard proteins yield the same peptide maps when extracted from Coomassie- and silver-stained gels, as judged by electrospray and MALDI mass spectrometry. The low nanogram range can be reached by the protocols described here, and the method is robust. A silver-stained one-dimensional gel of a fraction from yeast proteins was analyzed by nanoelectrospray tandem mass spectrometry. In the sequencing, more than 1000 amino acids were covered, resulting in no evidence of chemical modifications due to the silver staining procedure. Silver staining allows a substantial shortening of sample preparation time and may, therefore, be preferable over Coomassie staining. This work removes a major obstacle to the low-level sequence analysis of proteins separated on polyacrylamide gels.

In biological research proteins are now most commonly isolated or assayed by one-dimensional or two-dimensional gel electrophoresis. The proteins are subsequently visualized by staining, often by Coomassie Brilliant Blue if the protein is abundant enough (i.e., more than 100 ng). By electroblotting and digesting the protein on a membrane or by digesting it directly in gel, peptides are produced which are then extracted and analyzed. This analysis most commonly consists of Edman degradation of the HPLC-separated peptides. The goal is to obtain sequence information to identify the protein in sequence databases or to clone the corresponding gene.[1−3] Mass spectrometry has recently begun to be employed in this scheme, typically for mass analysis of the separated peptides[4−7] but also for obtaining peptide mass maps to be used in database searches.[8−13]

Visualization by Coomassie staining is tedious and can interfere with subsequent analysis steps. First, the gel is stained for 2−3 h, and then the gel background is destained overnight to visualize the proteins. To promote efficient enzymatic cleavage and to reduce the background for subsequent mass spectrometric analysis, any excessive Coomassie must be carefully removed. After a protein spot has been excised, additional long washing steps are necessary to remove Coomassie associated with the protein. The staining and destaining slows down the procedure and limits its throughput, and the extensive washing steps could lead to protein losses.

Recently, electrospray mass spectrometry[14−16] and matrix-assisted laser desorption mass spectrometry[17,18] have reached extremely low detection limits in the analysis of isolated peptides. For example, Emmett and Caprioli[19] have demonstrated low attomole detection limits with electrospray, and Vorm et al.[20] have obtained the same with matrix-assisted laser desorption/ionization (MALDI). Subpicomole detection and sequencing of proteins from polyacrylamide is thus a realistic proposition. In our laboratory, we have developed a nanoelectrospray ion source (NanoES),[21,22] which we have used for the sequencing of proteins from polyacrylamide gels.[23] In the course of that work, it became apparent that the 50−100 ng detection limit of the Coomassie staining method was not low enough for the levels that could be reached.

Silver staining is a popular and more sensitive staining method, with a detection limit between 1 and 10 ng.[24,25] The use of silver staining, if possible, would address the two problems mentioned

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above. It would decrease the amount of gel workup by eliminating washing steps because, in contrast to staining by dyes, silver staining does not act by specific binding to the protein.[26] Even more importantly, it would lower the protein amount needed for the sine qua non step of protein visualization and provide the means for mass spectrometry to improve sensitivity down to a few nanograms of protein material on the gel.

Several well-established protocols for silver staining have been published (see, for example, refs 24, 27, and 28 and references cited in ref 25). The major concern in applying the silver staining technique when followed by protein microanalysis is that it includes protein treatment with the strong oxidizing agent Ag^+. This is generally thought to cause oxidative attack on the protein, leading to chemical modification or destruction, hampering subsequent primary structure characterization. Moreover, several published protocols require various sensitizing pretreatments of the gel with glutaraldehyde, chromic acid, sodium thiosulfate, etc., which could also result in covalent modifications of proteins. While the microanalysis of silver-stained proteins was thus generally considered impossible, we failed to find any systematic description of the above-mentioned interfering processes. We are also not aware of any reports of the application of silver staining in protein microsequencing projects.

In this paper, we establish the feasibility of the microcharacterization of silver-stained proteins by MALDI and by NanoES tandem mass spectrometry. Model studies were performed on bovine serum albumin, because it is a much used standard in protein chemistry which has an intermediate size and presents some challenge because of its 17 disulfide bridges. The practical applicability and robustness of the protocol is demonstrated by the tandem mass spectrometric characterization of eight protein bands from a one-dimensional silver-stained polyacrylamide gel of an affinity preparation of proteins from budding yeast.

EXPERIMENTAL SECTION

Materials and Reagents. Bovine serum albumin was from Sigma Chemical Co. (St. Louis, MO). Its sequence was taken from SWISSPROT, accession no. P02769, without the signal and propeptide (amino acids 1−24). The concentration of the stock solution was determined by amino acid analysis. Except when otherwise noted, all the chemicals used, including α-cyano-4-hydroxy-*trans*-cinnamic acid for MALDI matrix preparation, were purchased from Sigma and were analytical grade, except silver nitrate, which was SigmaUltra grade. Nitrocellulose was from Bio-Rad (Richmond, CA). MilliQ water (Millipore, Bedford, MA) was used to prepare silver and Coomassie staining solutions. For mass spectrometric analysis and gel spot preparation, HPLC grade water, methanol, and acetonitrile (LabScan, Dublin, Ireland) were used.

One-dimensional SDS−polyacrylamide gel electrophoresis was performed using standard methods on the Bio-Rad Mini-Protean II system (7 cm × 10 cm minigels). 12% acrylamide gels of 0.5 or 1 mm thickness were used in model experiments. Some gels, where specified, were stained with 0.2% Coomassie Brilliant Blue R250 in 50% MeOH in water containing 2% acetic acid for 1 h (which also fixes the proteins) and destained overnight with the same solvent, excluding the dye.

The sample of yeast proteins was obtained from a collaborating group in the cell biology program, EMBL (Group Leader, Tony Hyman). It represents a fraction from a budding yeast protein preparation eluted from an affinity column with immobilized DNA. This fraction was subjected to one-dimensional electrophoresis on a 0.75 mm, 8% acrylamide gel.

Silver Staining. Silver staining was performed similarly to the method described in refs 27 and 28, except that treatment with glutaraldehyde (a cross-linking and sensitizing agent) was omitted. After electrophoresis, the gel slab was fixed in 50% methanol, 5% acetic acid in water for 20 min. It was then washed for 10 min with 50% methanol in water and additionally for 10 min with water to remove the remaining acid. The gel was sensitized by a 1 min incubation in 0.02% sodium thiosulfate, and it was then rinsed with two changes of distilled water for 1 min each. After rinsing, the gel was submerged in chilled 0.1% silver nitrate solution and incubated for 20 min at 4 °C. After incubation, the silver nitrate was discarded, and the gel slab was rinsed twice with water for 1 min and then developed in 0.04% formalin [35% formaldehyde in water (Merck, Darmstadt)] in 2% sodium carbonate with intensive shaking. After the developer turned yellow, it was discarded and replaced with a fresh portion. It is essential that the developing is carried out in an absolutely transparent solution. After the desired intensity of staining was achieved, the development was terminated by discarding the reagent, followed by washing of the gel slab with 5% acetic acid. The complete staining procedure lasts no longer than 1 h. Developed gels were completely transparent when the sensitization step with sodium thiosulfate was included. Silver-stained gels were stored in a solution of 1% acetic acid at 4 °C until analyzed.

In Gel Digestion. Protein spots were excised from the gel and in gel digested with trypsin according to published procedures[29,30] as modified by us.[23] A "control" piece of gel was cut from a blank region of the gel and processed in parallel with the sample. In contrast to the protocol described for Coomassie-stained gels,[23] all prewashing steps were omitted. After the gel pieces were excised and shrunk by dehydration in acetonitrile, which was then removed, they were dried in a vacuum centrifuge. A volume of 10 mM dithiotreitol (DTT) in 100 mM NH_4HCO_3 sufficient to cover the gel pieces was added, and the proteins were reduced for 1 h at 56 °C. After cooling to room temperature, the DTT solution was replaced with roughly the same volume of 55 mM iodoacetamide in 100 mM NH_4HCO_3. After 45 min incubation at ambient temperature in the dark with occasional vortexing, the gel pieces were washed with 50−100 μL of 100 mM NH_4HCO_3 for 10 min, dehydrated by addition of acetonitrile, swelled by rehydration in 100 mM NH_4HCO_3, and shrunk again by addition of the same volume of acetonitrile. The liquid phase was removed, and the gel pieces were completely dried in a vacuum centrifuge. The gel pieces were swollen in a digestion buffer containing 50 mM NH_4HCO_3, 5 mM $CaCl_2$, and 12.5 ng/μL of trypsin (Boehringer Mannheim, sequencing grade) in an ice-cold bath. After 45 min, the supernatant was removed and replaced with 5−10 μL of the same buffer, but without trypsin, to keep the gel pieces wet during enzymic cleavage (37 °C, overnight). Peptides were extracted by one change of 20 mM NH_4HCO_3 and three changes

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of 5% formic acid in 50% acetonitrile (20 min for each change) at room temperature and dried down.

Sample Preparation for MALDI Analysis. Microcrystalline matrix surfaces were made on the probe tips of the mass spectrometer according to the sample preparation procedure described in detail previously,[20] modified as outlined below.

The matrix material (α-cyano-4-hydroxy-*trans*-cinnamic acid) and nitrocellulose were dissolved in acetone/2-propanol (1:1 v/v) to a concentration of ~20 and 5 g/L, respectively. Approximately 0.5 μL of the matrix/nitrocellulose solution was deposited on a stainless steel sample stage, where it spread rapidly, allowing fast evaporation of the solvent.[31] Aliquots of 0.5 μL of analyte solution (identical to those used for ESMS before desalting/concentration) were deposited onto these matrix surfaces, and the solvent was allowed to evaporate at ambient temperature. Samples were rinsed by placing a 5−10 μL volume of water on the matrix surface after the analyte solution had dried completely. The liquid was left on the sample for 10 s and was then blown off by pressurized air. This washing procedure was repeated twice.

MALDI Mass Spectrometry. All mass spectra were obtained on a modified Bruker REFLEX mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany). The original data acquisition system was replaced by a LeCroy 9350AM 1 gigasample/s digital storage oscilloscope (LeCroy Corp., Chestnut Ridge, NY), from where single shot spectra were transferred to a Macintosh PowerPC 7100/80 computer (Apple Computer Inc., Cupertino, CA) via a National Instruments NI DAQ GPIB controller board (National Instruments, Austin, TX). Control of all data acquisition parameters and the transfer and subsequent averaging of time-of-flight data, as well as all further data processing, were carried out using the computer program LaserOne developed in our group. MALDI peptide spectra were calibrated using several matrix ion peaks as internal standards.

NanoES Mass Spectrometry. Needles for electrospraying were made with a micropipette puller (Model P-87 Puller, Sutter Instrument Co., Novato, CA) from borosilicate capillaries (Clark Electromedical Instruments, Pangbourme, Reading, England) and gold-coated in batches of about 20 in a vapor deposition instrument (SCD 020, Balzers, Wiesbaden, Germany).[22] Dried protein digests were redissolved in 10 μL of 5% formic acid and concentrated/desalted on a capillary similar to the spraying capillary which was packed with ~100 nL of POROS R2 sorbent (Perseptive Biosystems, Framingham, MA), essentially as described in ref 22. NanoES was performed on an API III (Sciex, Toronto, Canada) mass spectrometer as described in refs 21 and 32. Q_1 scans were performed with a 0.1 Da mass step. For operation in the MS/MS mode, Q_1 was set to transmit a mass window of 2 Da, and spectra were accumulated with 0.2 Da mass steps. Resolution was set so that fragment masses could be assigned to better than 1 Da. Since there was usually 1 h of measurement time per peptide mixture available, collision energy was tuned individually for each peptide to obtain the best possible MS/MS spectra.

Tandem Mass Spectra Interpretation and Database Searching. Using software developed in our group based on AppleScript (Apple) and BioMultiView (Sciex), the mass fragments with highest m/z in the Y″ ion series (nomenclature according to ref 33) in the MS/MS spectra of tryptic peptide were joined into a short sequence stretch. Together with the molecular weight information, they were assembled into a peptide sequence tag[32] and searched against a protein sequence database using PeptideSearch version 2.6.[9,34] All searches were performed against a nonredundant sequence database (nrdb, prepared daily by C. Sander's group at EMBL) containing presently more than 160 000 entries. The complete nrdb database was converted for use by PeptideSearch on a weekly schedule. No restrictions on protein molecular weight, p*I*, or species of origin were applied in the database searches.

After a protein match had been found with a peptide sequence tag, all ion series were used to confirm the identification with the complete MS/MS spectrum using BioMultiView. Each peptide match found in the database was verified in this way, and none was accepted on the basis of unique retrieval from the database alone. If there was no match for a particular peptide, an error-tolerant search was used. This type of search is described in ref 32. Briefly, a peptide sequence tag divides a peptide into three regions: the middle region, where the sequence has been determined, and the two regions at the N- and C-termini of the peptide. By only requiring the matching of two of the three regions, peptides with amino acid sequence differences between the actual peptide and the database entry and chemically or naturally modified peptides can be retrieved.

RESULTS AND DISCUSSION

The Silver Staining Procedure. Many silver staining protocols claiming roughly the same limit of detection (about 1−10 ng) have been reported (see ref 26 and references cited in ref 25). Briefly, proteins separated on polyacrylamide gels are fixed, and then gels are treated with the sensitizers—substances known to enhance the contrast between the stained protein bands and background, thus improving the sensitivity. Sensitizers act via different chemical mechanisms—increasing the binding of silver (sulfosalicylic acid), creating latent images of bands by the precipitation of microgranules of silver sulfide (sodium thiosulfate, DTT), promoting silver reduction (glutaraldehyde), complexing free silver cations not bound to a protein (chelators), etc.[25,26]—and their application is optional. After sensitization, the gel is exposed to silver nitrate solution, and silver ions complex with the proteins. In the subsequent developing stage, which is similar to photographic developing, the silver ions complexed with the protein undergo faster reduction than free silver ions. Particles of colloid silver (between 20 and 80 nm[35]) then form preferentially at the protein bands at the two surfaces of the gel, making them visible.

We chose the so-called "acidic" variant in Rabilloud's classification of silver staining methods,[26] in which the gel is treated with silver nitrate solution at neutral or weakly acidic pH. In contrast to most methods, we omitted the fixation/sensitization treatment with glutaraldehyde, which is known to attach covalently to the protein through Schiff base formation with ε- and α-amino groups. We were concerned that these Schiff bases would later not be hydrolyzed quantitatively, especially since glutaraldehyde can readily produce cross-linked oligomers.[26] According to several published silver staining protocols, the glutaraldehyde sensitization step is not crucial for obtaining detection limits in the low nanogram range (see also below and Figure 3).

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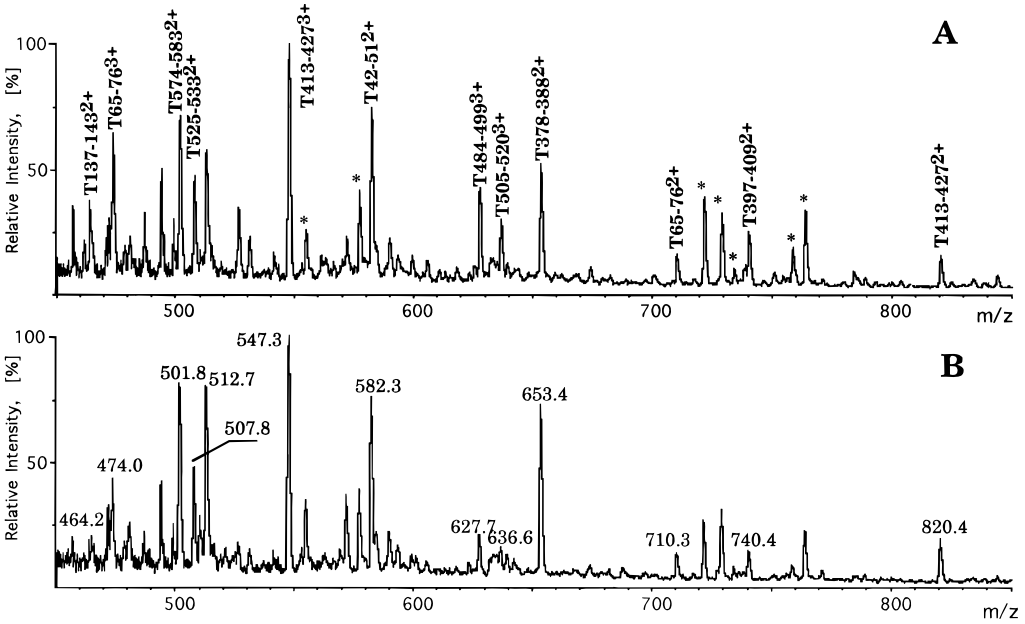

Figure 1. Comparison of the tryptic peptide maps of Coomassie- and silver-stained BSA. Samples of 0.8 pmol of BSA were applied to two wells of a one-dimensional polyacrylamide gel. One of the lanes was silver-stained, and the other was Coomassie-stained. After processing, the two samples resulted in the NanoES spectra shown in A (silver-stained) and B (Coomassie-stained). All labeled peaks have been identified by tandem mass spectrometry. The peaks marked by asterisks are from tryptic autodigestion. The figure shows near identity between the peptide maps obtained by the two staining methods.

The sensitization treatment with sodium thiosulfate was found necessary to obtain completely transparent gel background and was harmless toward protein molecules. In contrast to published methods, we performed the silver nitrate treatment at 4 °C in order to minimize oxidation reactions.

The detection limit of the staining procedure for BSA was 1.6 ng (25 fmol) applied to the gel. The procedure described here, therefore, appears to be at least as sensitive as protocols described by other workers.[25]

Compatibility with Protein Microcharacterization. To establish the compatibility of silver staining with microsequencing (as compared to Coomassie staining), the following questions need to be addressed: (a) whether any peptides can be recovered after digestion of silver-stained proteins, and if so, whether they are chemically modified and which chemical groups are the targets for this modification, and (b) whether the recovered peptides are the same ones as those recovered from a digest of Coomassie-stained protein.

To shed light on these questions, aliquots of 0.8 pmol of bovine serum albumin (BSA) were loaded into two wells of a minigel, and electrophoresis was performed. The gel was then cut into two parts, one of which was stained by silver and the other by Coomassie. The gels were processed as described in the Experimental Section and as in the previously established protocol,[23] respectively. NanoES mass spectra were obtained of the extracted peptide mixtures, and, as Figure 1 and Table 1 show, no differences could be detected in the resulting peptide maps. Even cysteine-containing peptides were detected in the *S*-acetamide-alkylated form (as confirmed by tandem mass spectrometry), proving that their cysteine groups had not been irreversibly oxidized or destroyed in some other manner.

To establish the compatibility of silver staining with MALDI mass spectrometry, a few percent of the same sample as used for electrospray was applied to a matrix surface (see Experimental Section). The resulting reflector spectrum (Figure 2) isotopically resolved all peptide peaks, and the standard deviation of the mass accuracy was better than 19 ppm (Table 1). The signal-to-noise ratio in the spectrum was even better than that in an analysis of an equivalent amount of Coomassie-stained protein, an observation which we have made in other analyses as well (data not shown). It has previously been shown that Coomassie can form intensive adduct peaks in the spectra of electroblotted proteins.[36] Thus, the improved signal-to-noise ratio in the analysis of silver-stained gels could be due to the absence of residual interfering dye still associated with the sample. Based on their masses, 34 tryptic peptides were assigned to the sequence corresponding to a coverage of almost two-thirds of the sequence. Very few peaks were observed that could not be assigned to the sequence or to known background. As in the investigation by NanoES, there was no evidence of chemical modification of BSA.

Next we investigated whether silver staining could lead to improved sensitivity for the overall procedure from detection of protein on the gel to final mass spectrometric analysis. For this purpose, 80 fmol of BSA was applied to a 1 mm gel. This protein amount was not readily visible after Coomassie staining but was detected as a sharp and clear band after silver staining (Figure 3A). The nanoelectrospray mass spectrometry analysis of the tryptic digest from this band in Q_1 scanning mode showed only trypsin autolysis peaks but no peptide peaks from the protein. However, a parent ion scan for the immonium ion of Leu or Ile[37] revealed four digestion products. Subsequent fragmentation of these ions at *m*/*z* 547.4, 582.5, 653.5, and 740.5 produced MS/MS spectra containing enough information for protein identification through database searching using peptide sequence tags[32] (Figure 3D).

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Table 1. Measured Peptides Masses from Figures 1 and 2

AA residues	calcd M_r (monoisomers)	ΔM (Da)[a]	MS/MS[b]	sequence[c]
466−471	659.35	−0.03		TPVSEK
132−136	664.37	0.01		KFWGK
212−217	688.37	0.01		AWSVAR
188−194	702.40	0.01		VLASSAR
5−10	711.37	0.04		SEIAHR
174−180	757.42	0.02		GACLLPK
233−239	788.46	0.02		LVTDLTK
428−435	816.48	−0.03		SLGKVGTR
538−544	817.42	0.02		ATEEQLK
205−211	819.46	−0.07		FGERALK
218−224	846.50	0.01		LSQKFPK
459−465	897.47	−0.05		LCVLHEK
181−187	905.46	0.04		IETMREK
137−143	926.49	0.02	X	YLYEIAR
209−217	1000.58	0.02		ALKAWSVAR
574−583	1001.58	0.03	X	LVVSTQTALA
525−533	1013.61	nf[d]	X	QTALVELLK
137−144	1082.59	0.03		YLYEIARR
564−573	1106.51	0.07		EACFAVEGPK
524−533	1141.71	0.02		KQTALVELLK
233−242	1152.68	nf[d]	X	LVDTLTKVHK
42−51	1162.62	−0.02	X	LVNELTEFAK
436−444	1165.49	0.03		CCTKPESER
1−10	1192.59	0.02		DTHKSEIAHR
378−388	1304.71	0.01	X	HLVDEPQNLIK
534−544	1307.72	−0.07		HKPKATEEQLK
65−76	1418.69	0.00	X	SLHTLFGDELCK
336−347	1438.80	0.00		RHPEYAVSVLLR
52−64	1462.58	0.06		TCVADESHAGCEK
397−409	1478.79	−0.03	X	LGEYGFQNALIVR
274−285	1531.77	0.01		LKECCDKPLLEK
459−471	1538.81	−0.01		LCVLHEKTPVSEK
323−335	1566.74	−0.01		DAFLGSFLYEYSR
413−427	1638.93	−0.05	X	KVPQVSTPTLVEVSR
323−336	1722.84	−0.06		DAFLGSFLYEYSRR
445−458	1723.83	−0.05		MPCTEDYLSLILNR
484−499	1879.91	−0.04	X	RPCFSALTPDETYVPK
505−520	1906.91	0.07	X	LFTFHADICTLPDTEK
317−335	2300.07	−0.03		NYQEAKDAFLGSFLYEYSR
389−409	2528.21	−0.05		QNCDQFEKLGEYGFQNALIVR

[a] Calculated minus measured mass from the MALDI spectrum in Figure 2. [b] NanoES tandem mass spectrometry was performed of the peptides marked in this colum (shown in Figure 1). [c] C designates *S*-carbamidomethyl cysteine. [d] nf, not found.

The sensitivity of the MALDI technique was examined by applying 10% of the above-mentioned digestion mixture to a MALDI target and measuring a reflector spectrum. Mass accuracy and resolution were unchanged from Figure 2 and the sequence coverage was still 39%. Ten peaks that could not be correlated to the BSA sequence are apparent in the spectrum (marked by asterisks in the figure), four of which are known autodigestion products of trypsin and six of which could not be assigned. It is not unusual to observe unassignable peaks in MALDI spectra of peptide maps from gels, i.e., peaks that do not correspond to predicted peptide masses or easily explainable mass shifts thereof. To prove the feasibility of protein identification from MALDI peptide mapping of silver-stained proteins, even at this low nanogram level, we searched the nrdb database by the complete peptide map and clearly identified BSA.

Sequencing of a Large Number of Peptides. Sequencing of many peptides could demonstrate the general applicability of the silver staining technique both by robust and practically applicable protein sequencing from silver-stained gels and by the absence of modifications induced by the staining. In a biological project involving yeast proteins (collaboration with T. Hyman, EMBL), we frequently identify many proteins from one-dimensional or two-dimensional polyacrylamide gels. One such gel—a separation of a fraction of affinity-purified proteins from budding yeast—is presented here as an example. The silver-stained, one-dimensional polyacrylamide gel shown in Figure 4 reveals a complex mixture, containing proteins with molecular mass in the range of 30−250 kDa and quantities from the low nanogram to the 1 μg range. Several bands of interest for the biological project were excised and processed as described in the Experimental Section.

As a tool to investigate the processed protein samples, we choose sequencing by NanoES tandem mass spectrometry, which has proven to be a powerful tool in protein identification projects.[23] This technique is well suited to high-sensitivity sequencing because of its robustness and speed and because there is no need for chromatographic separation of the peptide mixture. NanoES tandem mass spectrometry allows not only determination of modified peptides in unseparated digests but also determination of the nature of the modification and localization of its site.[37−39] Previously, we have demonstrated that the sensitivity of the NanoES ion source on our triple quadrupole instrument for sequencing is in the 5−10 fmol/μL range for peptide mixtures. This means that, with an overall yield of the protocol of even 20%, it should be possible to analyze peptide mixtures derived from 25−50 fmol of protein loaded on a gel, compatible with the sensitivity of silver staining. In practice, the problem is not in the absolute sensitivity of the machine but mostly in the ability to recognize the peptides in the chemical noise. In the case of NanoES, the signal-to-noise ratio can be increased by parent ion scans for the selective detection of peptides in complex mixtures,[37] as already explained in the model experiments on BSA.

All eight protein bands excised from the silver-stained gel were successfully identified, and the results are presented in Table 2. Since no limitation was placed on protein molecular weight in the database search, the coincidence of protein molecular weights only supports the validity of their identification. As explained in the Experimental Section unique retrieval of a protein on the basis of a peptide sequence tag was not accepted as proof of the correctness of an identification. Rather, the retrieved peptide sequence had to match the complete MS/MS spectrum and not only the short part used to construct the sequence tag. In this matching scheme, the presence of all-ion series (A, B, Y″, and B series starting from internal proline) and the low abundance of some fragment ions (such as those from cleavage C-terminal to a proline) are taken into account. Positive identification of a protein was made on the basis of at least three peptides sequenced in this way, and at least 40 amino acids were covered in the protein: 101 amino acids per protein on average. [Disregarding the case of one protein which was present as a trace in a mixture of other proteins and of which only one peptide appeared (see Table 2)]. We would like to point out that the identification of a protein in such a way is certain and not merely very probable. Gene families and splicing variants (i.e., proteins that differ because of different mRNA processing) can still lead to multiple matches, but the identity of the peptides sequenced is certain.

Against the backdrop of this certain identification, we were surprised by the results from bands 5 and 7, which were identified as virus proteins instead of proteins from yeast. However, retrieval

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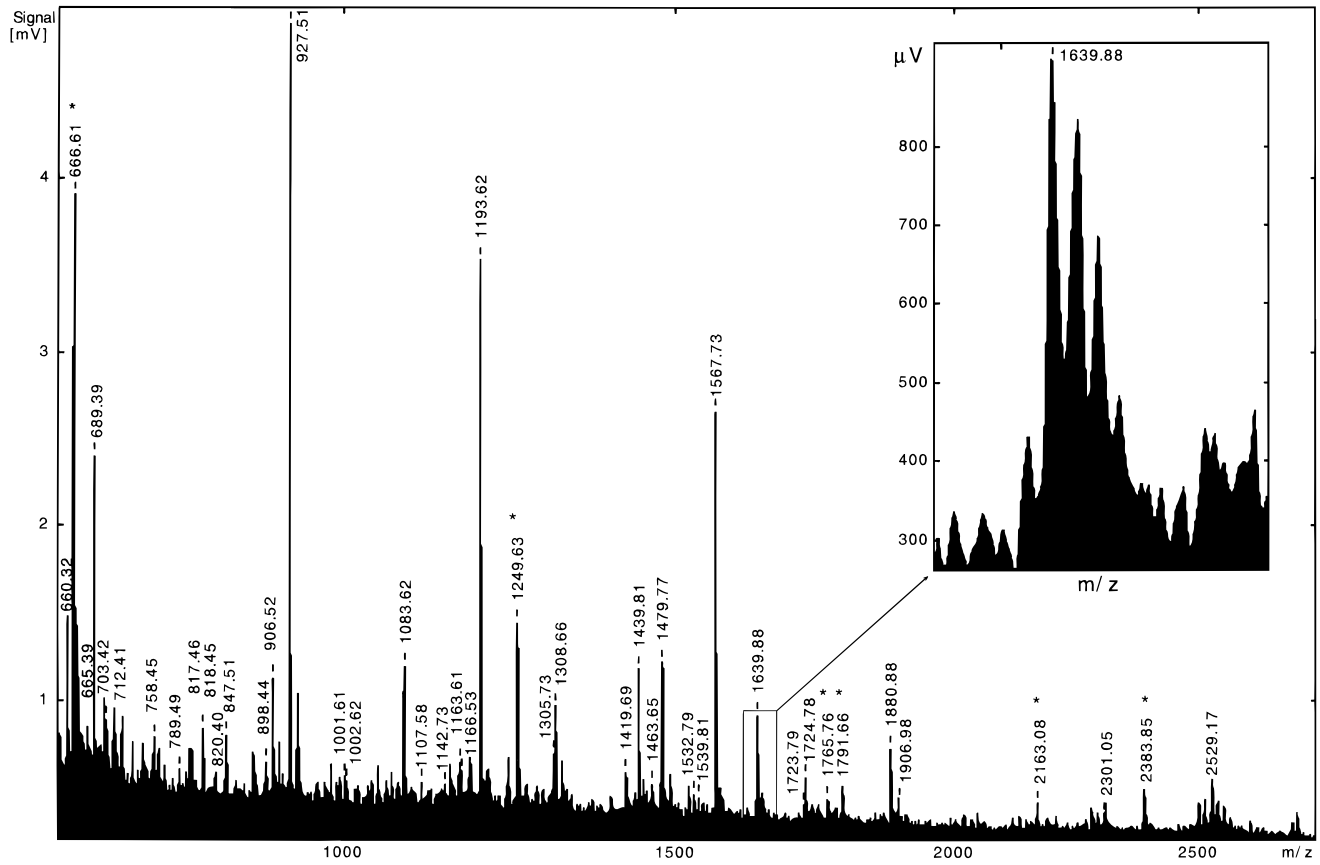

Figure 2. MALDI reflector mass spectrum of the tryptic peptide mixture of 0.8 pmol of BSA applied to a gel. A volume of 0.5 μL of ~25 μL of peptide solution was applied to the MALDI target. The insert shows the resolution of about 2400 fwhh obtained on the peptides. All assigned masses correspond to tryptic peptides within 50 ppm, and the standard deviation of the mass error is 19 ppm (see Table 1). Peaks additionally marked by asterisks are from tryptic autodigestion products or other background ions. For further discussion, see text.

of information about the matches found identified the two proteins as originating from *Saccharomyces cerevisiae* virus L-A , a nonenveloped, double-stranded RNA virus known to infect *S. cerevisiae* cultures. Since the genome of this virus is not a part of the yeast genome, these proteins were identified only because the peptide sequence tags were searched against the inclusive database, not one restricted to certain species. As shown in Table 2, other non-yeast proteins—human keratins—were identified as well.

Band 7 also illustrates another interesting issue in protein identification. The protein found has not been biochemically characterized but was only known as an open reading frame, that is, a region of DNA derived from genomic sequencing which appeared to code for a protein. Thus, it seems that this protein has been observed for the first time in this experiment.

Band 4 (see Table 2) was found to contain three proteins that were all reliably identified. Reinspection of other lanes of the gel revealed a faint additional band just above the main band. Thus, the technique of sequencing by NanoES and identification by peptide sequence tags is not limited to isolated proteins but can deal with unseparated protein mixtures as well, a common occurrence in one-dimensional gels. When sequencing proteins at very low levels, as in the context of silver-stained gels, protein mixtures are the rule rather than the exception. As an example, the human keratins mentioned above which originate from chemicals and/or sample handling often become ubiquitous at these low levels. We have found that the actual pattern of keratin peptides is not predictable, so that they cannot be subtracted using a control.

Three peptides from bands 5 and 8 could not be assigned to peptides in the database by a first database search using the peptide sequence tag approach. To check whether they were possible oxidation products due to the silver staining, it was important to elucidate their origin. Error-tolerant database search—looking for a matching of only two of the three regions of a peptide sequence tag—allows the identification of peaks whose mass does not fit any of the expected peptides present in the database. (Note that common modifications such as oxidized methionine and acrylamidated cysteine were taken into account in the search directly, by a modified molecular weight of these amino acids.)

The following case serves as an illustration of the procedure. In the peptide map of band 5, we observed an intensive doubly charged peak at *m/z* 685.9 (Figure 5A). A series of ions assumed to be Y″ ions, which specified the partial sequence **EVS**, was quickly and unambiguously retrieved from the MS/MS spectrum of this peak as described in the Experimental Section (Figure 5B). From this single spectrum, we could not directly extend the partial sequence to the C-terminus, since a large number of low-intensity peaks resulted in too many ambiguities to continue the Y″ ion series. Searching against the nrdb database with the sequence tag (870)SVE(1184.6) and neutral monoisotopic peptide mass 1369.8 Da retrieved no matches. Error-tolerant searching matched a partial peptide from the same virus protein (SVEVS). This N-terminal sequence was confirmed in the spectrum by the B ion series. The corresponding tryptic peptide SVEVSTTIYDTHVQAGAHAVYHASR, however, has the mass 2688.3 Da, indicating that

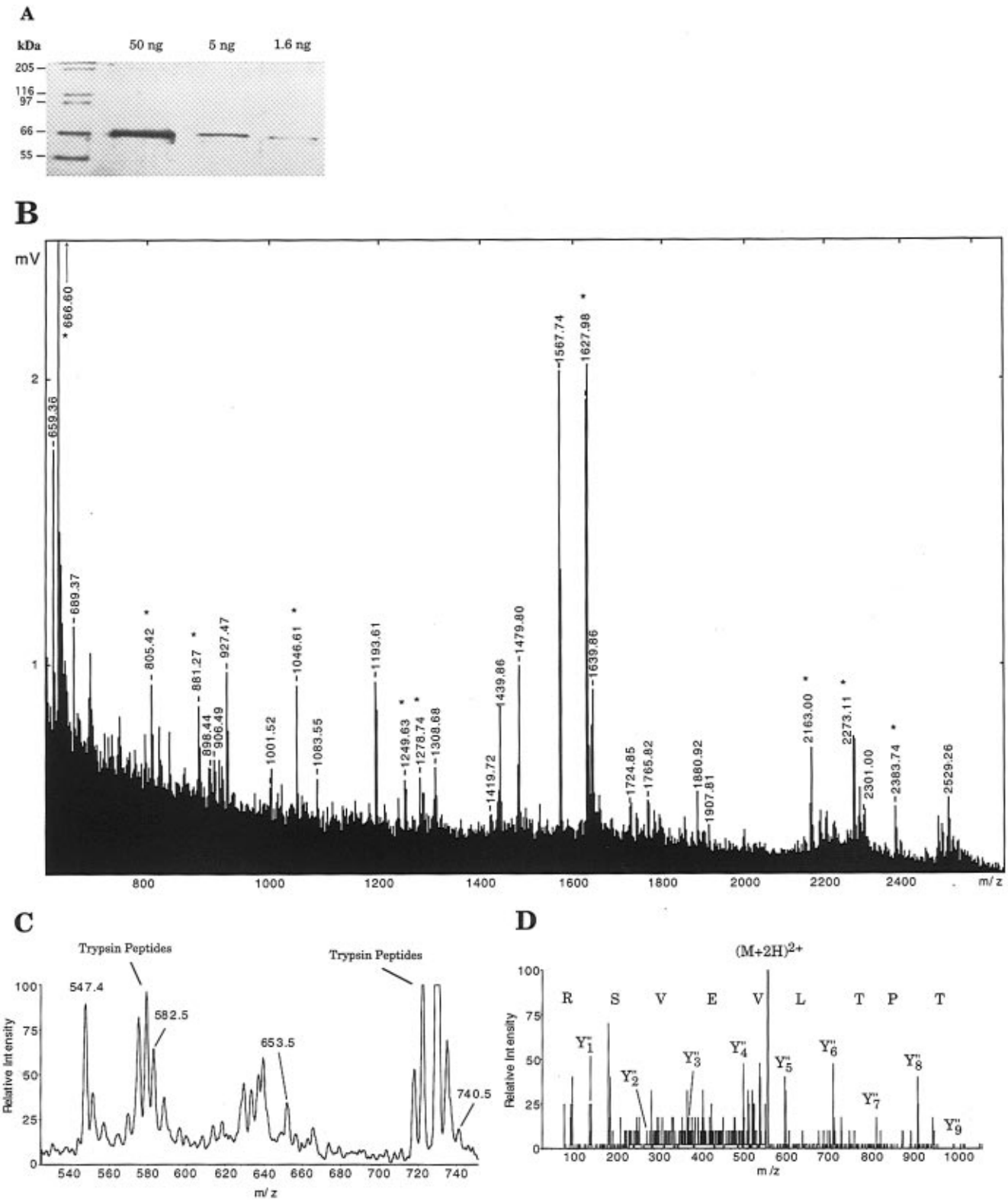

Figure 3. Sensitivity of mass spectrometric methods demonstrated on 80 fmol of BSA (5 ng) applied to a one-dimensional polyacrylamide gel. (A) Scanned picture of the silver-stained gel with three different amounts of BSA loaded. The 1.6 ng (25 fmol) lane can still be detected, showing good sensitivity of the staining procedure. (B) MALDI reflector mass spectrum of the tryptic peptide mixture of the 80 fmol lane; 10% of the extracted peptide mixture was applied to the target. Mass accuracy and resolution were as high as in Figure 2. The main differences from Figure 2 are a lower sequence coverage (39 vs 62%), a slightly lower signal-to-noise ratio, and the appearance of more peaks that do not correspond to the BSA sequence (Trypsin autolysis products and other background ions, marked by asterisks). (C) Parent ion scan for the immonium ion of isoleucine/leucine (*m/z* 86) on the extracted peptides from lane 4 in A (5 ng). The labeled peaks have been sequenced by tandem mass spectrometry. (D) Tandem mass spectrometric analysis of the peak with *m/z* 547.4 in C.

an additional cleavage site should be present in the sequence and that it may be the reason for the difference between the sequence of the peptide and the sequence in the database. Since Arg or Lys should be the C-terminus of the peptide, we subtract their masses from the measured mass of the peptide. The obtained mass is found as an N-terminal piece of the retrieved sequence,

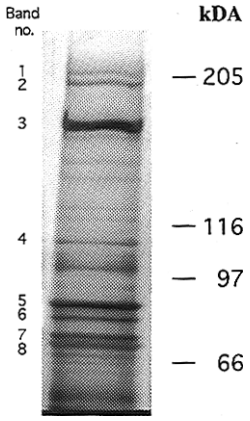

Figure 4. Scanned picture of a silver-stained gel from a one-dimensional gel separation of a fraction of yeast proteins. The bands used in the subsequent mass spectrometric analysis are marked.

namely SVEVSTTIYDT**R**, and this sequence indeed fits the tandem mass spectrometric data. One point mutation or a single mistake in a nucleotide sequence can explain the observed sequence difference of R versus H.

Two other cases of inconsistency with the database sequences were found in the heat shock protein (band 8) (data not shown). The method of error-tolerant searching was applied essentially as described above. All three cases of disagreement between found sequences and ones retrieved from a database could be ascribed to the sequence, and in no case could they be associated with an oxidative destruction processes.

Absence of Chemical Modifications. The sequencing encompassed more than 80 peptides covering more than 1000 amino acid residues. A representative range of amino acid compositions including all amino acids was thus present in these peptides. Even peptides containing amino acid residues highly susceptible to chemical modification were identified in their native form, and in none of the peptides did we observe any indications of protein oxidative destruction. Cysteine-containing peptides (four in four proteins) were detected in an acetamide-alkylated form or (in one case) in an acrylamide-modified form. Since alkylation was performed after staining, it appears that no cysteine oxidation to cysteic acid or desulfurization took place during

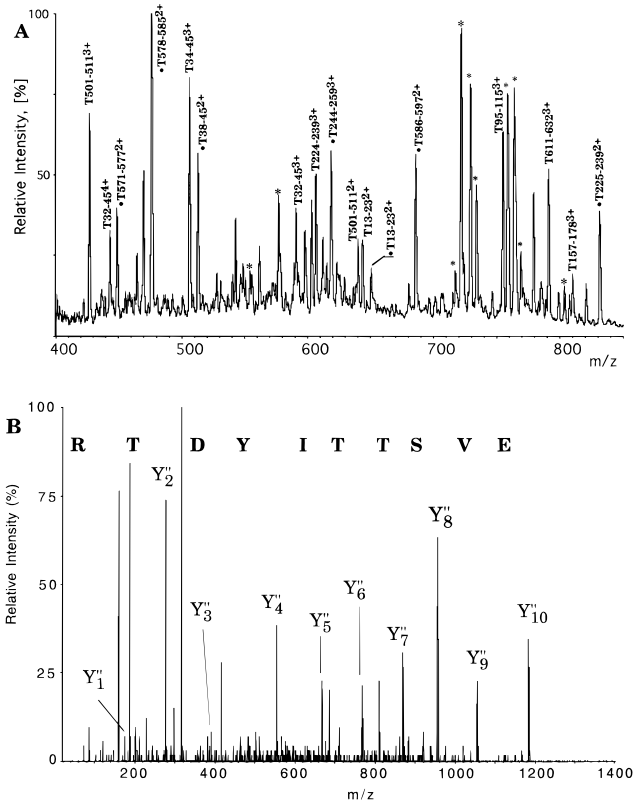

Figure 5. (A) Peptide map of band 5 from the gel in Figure 4. Peaks marked with a bullet were sequenced and used for a database search. (For identification see Table 2.) Peaks marked with asterisks are tryptic autolysis products. (b) Tandem mass spectrum of the ion with *m/z* 685.9 (peptide $T_{568-597^{2+}}$ in Figure 5a) used for error-tolerant searching as discussed in the text.

treatment with silver nitrate. Eight methionine-containing peptides were found in five proteins. Five were detected in the native form, two were found in both the native and sulfoxide forms, and one was found in the oxidized form only. Partial oxidation of methionine residues is, in our experience, frequently observed in the analysis of polyacrylamide gel-isolated proteins and has also been reported by other groups.[40,41] All eight tryptophan-containing peptides in five proteins were observed in their native form.

Table 2. Identification of Yeast Proteins in the Fraction Eluted from the Affinity Column, Separated by One-Dimensional Polyacrylamide Gel and Visualized by Silver Staining

band no.[a]	apparent mol mass, kDa	protein identified	acc. no.[b]	MW, calcd	no. of peptides sequenced	no. of amino acids covered
1	210	fatty acid synthase, subunit α	P19097	208	11	128
2	205	fatty acid synthase, subunit *β*	P07149	228	14	175
3	180	clathrin, heavy chain	P22137	187	12	164
4	110	α-ketoglutarate dehydrogenase	P20967	114	3	40
		phosphofructokinase 1	P16861	108	3	43
		vesicular traffic control protein SEC15	P22224	105	1	10
5	80	*S. cerevisiae* virus L-A major coat protein	P32503	76	7	86
6	75	vacuolar sorting protein 1	P21576	78	9	118
7	70	gene, "cap"; product, "capsid"; *S. cerevisiae* virus L-A complete genome	U01060	78	3	55
8	70	heat shock protein SSA1	P10591	70	8	124
		contaminating human keratins [c]	P04264	57−65	6	74
			P12035			
			P13645			
			P35908			

[a] Band numbers correspond to the gel in Figure 4. [b] Accsession numberes correspond to SwissProt database, except no. 7, which corresponds to GenPept. [c] Human keratins were found as the minor impurities in all the fractions analyzed.

MALDI analysis of several of the proteins (data not shown) also confirmed the above findings.

All the results obtained in the present study show that no considerable oxidative damage occurs during silver staining of proteins in polyacrylamide gels. This result was unexpected from common belief[42] but is in agreement with what is known about the mechanism of the silver staining process.[26] Our results suggest that no "primary" protein oxidative destruction is necessary to generate the nucleation centers for the formation of colloidal silver granules, visualizing the protein bands. Protein oxidative modification, if it occurs at all, seems to be a minor side reaction and not a main process that could be responsible for image development or for determining the detection sensitivity.

CONCLUSION AND PROSPECTS

The above results clearly prove that silver staining (at least in the form used here) is compatible with microanalytical protein characterization because it does not introduce chemical modifications. Even side chains known to be quite unstable toward oxidation, such as the −SH groups of cysteine, methionine, and the tryptophan side chain, "survive" treatment with silver ions. Results we have obtained in similar studies with zinc−imidazole reverse staining using the method of Fernandez-Patron[42,43] yielded essentially the same results as silver staining.

Mass spectrometric microsequencing of proteins at the silver-stained level pointed out some specific advantages of the Nano ES MS/MS technique combined with protein identification with peptide sequence tags. Protein sequencing on this level is often a sequencing from mixtures (e.g., with human keratins), where the target protein is not always a major component. Thus, the possibility of sequencing protein mixtures is a vital point when dealing with proteins in the silver staining range. The analysis of proteins at low levels often rests on a small number of detected peptides. This means that it is not practical to split the collected data into acceptable and nonacceptable spectra and to use only the former ones for the interpretation and protein identification. Particularly for samples in low amounts, this underlines the usefulness of the sequence tag approach as compared to approaches based on complete spectrum interpretation.

As we have demonstrated, silver staining has considerable advantages compared to the traditional Coomassie staining technique. Not only is the detection limit nearly 100 times lower than that obtained with Coomassie staining, but the sample preparation time is shortened and the total procedure results in less background. Thus, we recommend that silver staining be widely used in microanalytical work.

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