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In-Gel Digestion of Proteins Using a Solid-Phase Extraction Microplate

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We present a new procedure for in-gel digestion of proteins introducing a combination of two different 96-well microplates. The two plates have incorporated small capillaries with a length of 2.4 mm in each well, one of which has 75-μm-inner diameter capillaries, whereas the second plate has reversed-phase-type capillaries fixed to it. The initial steps of the in-gel digestion process, comprising destaining, reduction/alkylation, dehydration, and digestion, was carried out in the plate containing 75-μm capillaries. Capillaries containing C18 reversed-phase modified monolithic silica rods of a 200-μm diameter were used for the second plate in which extraction and cleanup of peptides were carried out. Peptides were eluted directly from the solid-phase extraction plate onto the MALDI sample support. The separation of the process into two plates led to increased process stability, without compromising sensitivity, i.e. peptide recovery, making it suitable for true high-throughput protein identification. The handling of proteinases could easily be optimized, and no restrictions were made on chosen pH range through the absence of the solid phase in the initial steps of the protocol. Efficient binding of peptides to the solid phase and subsequent direct elution onto the MALDI sample support led to sensitivities in the attomole range. Performance of the process was demonstrated with tryptic digests of proteins stained with colloidal coomassie blue, silver, and the fluorescent stain SYPRO Ruby.

Mass spectrometry has developed into a significant enabling technology for proteomic analyses.[1−9] The current generation of mass spectrometry instruments has steadily improved in sensitivity and throughput. In particular, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) allows sample supports with large number of samples to be introduced into the instrument and analysis of each sample to be performed within seconds. Current sensitivity levels extend into the very low attomole range.[10]

The enzymatic digestion of proteins in gels is a standard procedure in the field of proteomics.[11−13] Gel pieces are excised from 1D or 2D gels. A destaining of the gel pieces may be carried out prior to the digestion, which is usually done using trypsin. Digestion is stopped and the formed peptides are solubilized in acidic medium. The peptide mixture may then be concentrated and peptides purified before the sample preparation for mass spectrometric analysis. For gel pieces from 1D gels, reduction and alkylation steps are normally included in the process.

A large number of protocols have been published for the in-gel digestion process. They do all apply but differ in their sensitivity as well as the throughput that can be achieved with the methods. To cope with the dimensions of present and future proteomics projects, a prerequisite is the development of present in-gel digestion processes into high-throughput methods with sensitivity levels approaching those of the mass spectrometers.[14−16] In addition, the process should be highly flexible to deal with different staining techniques used for the visualization of proteins in gels.

Solid-phase extraction (SPE) is an established method for the purification and concentration of mixtures of peptides resulting from a proteolytic digest.[17] A sample cleanup is in general necessary before mass spectrometric analysis in order to remove interfering salts and small molecules. Sample concentration allows larger amounts of peptides to be placed on the mass spectrometric sample support thereby increasing sensitivity. Currently, pipet tips containing a small bed of C18 functionalized sorbent are widely used.[18−20] They are convenient for low-throughput applications but

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offer somewhat low dependable tip-to-tip performance. A 96-well plate with a low peptide binding membrane in which the first steps of the in-gel digestion process are carried out has been made available to be used in combination with the pipet tips. However, low recoveries were observed with this plate.[21]

Monolithic chromatographic supports have become available for HPLC applications.[22−25] Monoliths are advantageous over conventional porous particles because of defined micro-/macroporous structure leading to superior mass transfer. The monolithic supports can be manufactured with diameters in the low-micrometer range, and the microscopic structure can be controlled to optimize flow rates. Modifications can be introduced to control hydrophobicity.[25] The high performance and robust nature of these capillaries make them ideally suited for the cleanup and concentration of samples in the in-gel digestion process.

In this paper, we demonstrate a novel way of performing the in-gel digestion process including simultaneous cleanup and concentration of samples as well as the direct transfer to the MALDI sample support. Two new types of 96-well microplates are introduced, an SPE plate integrating capillaries containing monolithic solid phases for efficient binding and elution of peptides and a processing plate containing empty capillaries with a narrow diameter to provide optimal performance of the initial part of the process. A detailed description of the procedure is given, and examples showing the performance of the system are presented.

EXPERIMENTAL SECTION

Microplate Design and Automation. The processing plate used is a 96-well V-shaped polypropylene plate. At the bottom of each well, empty capillaries with a length of 2.4 mm and an inner diameter of 75 μm are inserted. The SPE plate incorporates capillaries of 200-μm inner diameter. These capillaries contain a C18 reversed-phase modified Chromolith (Merck KGaA, Darmstadt, Germany), which is a monolithic chromatographic support made of a highly porous silica rod. The microstructure has been optimized to obtain flow rates suitable for vacuum applications. The plates have an additional polypropylene construction as shown in Figure 1 to protect the capillaries. This feature also determines the distance from the end of the capillary to the MALDI sample support in the direct elution step (see below). The application was performed on a Genesis Freedom workstation (Tecan, Männedorf, Switzerland) with on-deck incubators and dedicated hardware to handle the processing and SPE plates. A pipetting unit of eight needles transfers liquid to the plates, and liquid is removed by means of vacuum. The vacuum unit was designed to prevent droplet formation underneath the plate, thus excluding risk of contamination. The adapter for the direct elution on the MALDI sample plate is designed such that all 384 positions on the AnchorChip targets (Bruker Daltonik, Bremen, Germany) can be accessed.

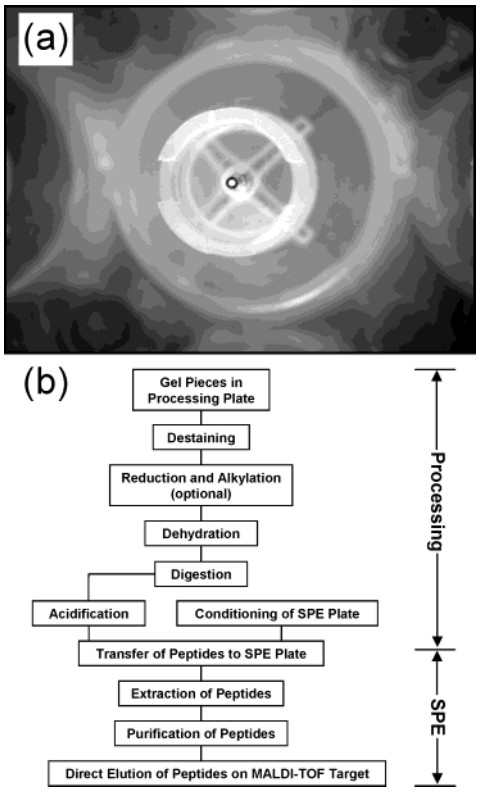

Figure 1. Image of section of the SPE plate (a) used for purification and concentration of peptides. The capillary contains the monolithic rod and is surrounded by the support for direct elution on MALDI-TOF targets. Gel pieces are placed in the processing plate for in-gel digestion of proteins, and generated peptides are transferred to the SPE plate for further processing (b).

Validation of the SPE Plate. A dilution series in 0.1% TFA of standard peptide mixtures containing seven different peptides (Bruker Daltonik) was made. Aliquots were transferred to the wells of the SPE plate, resulting in final volumes of 4 μL and peptide amounts in the range of 0.1 amol to 500 fmol per peptide. A 4-μL aliquot 0.1% TFA was added before binding to the solid phase. Bound peptides were washed with 10 μL of 0.1% TFA and subsequently eluted directly onto a 600-μm AnchorChip target (Bruker Daltonik) using 2 μL of 0.09 g/L α-cyano-4-hydroxycinnamic acid in 90% acetonitrile, 0.1% TFA. The experiment was repeated two times using four samples of each concentration.

One- and Two-Dimensional Electrophoresis (1DE, 2DE) and Image Analysis. 1DE was performed according to Laemmli[26] from bovine serum albumin (BSA) and human serotransferrin solutions using the NuPAGE kit (Invitrogen, Carlsbad, CA). Gels were stained with colloidal coomassie blue (BSA) following standard procedures or silver (transferrin) according to Shevchenko et al.[2] 2-DE was performed according to Görg et al.[27] from yeast and liver protein extracts. In short, samples were diluted in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% ampholytes pH 3−10), sonicated in intervals for 10 min, centrifuged at 20000*g* (15 °C) and subjected to isoelectric focusing (pH

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4−7, 18 cm) using immobilized pH gradients followed by SDS−PAGE according to Laemmli. The protein spots of the 2D gels were displayed by standard staining procedures with colloidal coomassie blue (yeast) or SYPRO Ruby (liver). The corresponding gels were digitized with a FLA 5000 laser scanner (Raytest, Straubenhardt, Germany) or a GS 800 densitometer (BioRad, Hercules, CA). Image analysis was performed with Proteom Weaver (Definiens AG, Munich, Germany).

In-Gel Digestion. Gel pieces of 1.5-mm diameter were excised either manually or in case of the SYPRO Ruby gel using the GelPix spot cutter from Genetix. Gel pieces were placed directly in the processing plate and destained by two times incubation with 50 μL of 50 mM NH_4HCO_3 in 30% acetonitrile for 5 min at 37 °C with the exception of silver-stained gel pieces, where no destaining was carried out. Gel pieces were then provided in 50 μL of 50 mM NH_4HCO_3 in 30% acetonitrile, and proteins were reduced by the addition of 5 μL of 10 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in 50 mM NH_4HCO_3 in 30% acetonitrile followed by 30-min incubation at room temperature. A volume of 5 μL of 40 mM iodoacetamide in 50 mM NH_4HCO_3 in 30% acetonitrile was added, and alkylation was performed at room temperature for 30 min in the dark. For gel pieces from 2D gels, reduction and alkylation were included in the preparation of the gels and were not repeated in the in-gel digestion process. Gel pieces were washed thoroughly before dehydration with 80 μL of 80% acetonitrile for 10 min. The gel pieces were swollen in 4 μL of 12.5 ng/μL trypsin (Gold Standard, MS Grade, Promega, Madison, WI) in 5 mM Tris-HCl, pH 8.0, for 10 min, and excess trypsin solution was removed by means of vacuum. Prior to incubation for 2 h at 37 °C, 4 μL of 5 mM Tris-HCl, pH 8.0, was added to keep the gel pieces wet during enzymatic cleavage. Peptides were extracted by addition of 4 μL of 1% TFA and 15-min incubation. The solid phase in the SPE plate was conditioned with 50 μL of 80% acetonitrile, 1% TFA followed by 25 μL of 1% TFA. Extracted peptides from the gel pieces were transferred by means of vacuum to the SPE plate. Peptides were bound to the solid phase and washed with 10 μL of 0.1% TFA. Samples for MALDI measurements were prepared as described above. An overview of the process is shown in Figure 1.

MALDI-TOF-MS. Mass spectra of positively charged ions were acquired on a Bruker Ultraflex instrument (Bruker Daltonik) operated in the reflector mode. A total of 50 single-shot spectra was accumulated from each sample. The total acceleration voltage was 25 kV. In the case of the SYPRO Ruby-stained samples, MS/MS spectra were acquired using the integrated LIFT device in addition to the peptide mass fingerprints. Spectra were calibrated externally using a standard peptide mixture. The XMASS 5.1.5 and MS Biotools 2.2 software packages (Bruker Daltonik) were used for data processing.

Database Searching. For protein identification, the SwissProt database, release no. 40.28, was searched using the Mascot Software (Matrix Science Ltd., London, U.K.). The probability score calculated by the software was used as the criterion for correct identification. A mass deviation of 75 ppm was tolerated in the searches, and oxidation of methionine residues was considered a possible modification.

RESULTS AND DISCUSSION

The processing plate was used for the first part of the in-gel digestion process comprising destaining, reduction, alkylation, dehydration, digestion, and extraction of the peptides from the gel pieces. The capillaries in the processing plate were small enough to prevent evaporation but large enough so that liquid could be efficiently removed on a vacuum manifold. A prepierced rubber plate cover ensured that the wells were tight enough to work with very small volumes during digestion. The capillaries made it possible to transfer small volumes down to 5 μL, reliably and with no significant losses, to the SPE plate. Size of the gel pieces to be processed was less critical using the flow-through principle for liquid handling compared to the conventional liquid-in−liquid-out handling. The processing plate is stable at acidic as well as alkaline pH, and no limitations in assay design and optimization were introduced.

The SPE plate introduces the Chromolith (Merck) material[28−31] in a 96-well microplate allowing parallel and fast desalting and sample cleanup of peptide mixtures. The plate geometry was optimized to allow direct elution on MALDI-TOF sample supports. For the AnchorChip targets (Bruker Daltonik), dedicated hardware components were developed to provide access to all 384 positions on the target. The direct elution option increased throughput. In 3 h and 30 min, 96 samples were processed including 2 h of digestion. It has been shown that digestion time can be reduced to 30 min without compromising sensitivity and peptide yield.[32] This would reduce processing time of 96 samples to 2 h with the presented system.

The two-plate principle was introduced to circumvent inherent problems related to a one-plate process. Excess trypsin solution was easily removed after swelling of the gel pieces to lower trypsin autolysis, which leads, in turn, to a decrease of interfering trypsin autolysis products. Furthermore, elution of peptides from the solid phase was carried out using a 90% acetonitrile solution. During elution in the one-plate process, the gel pieces come in contact with the acetonitrile solution and aqueous solution is extracted from the gel pieces. Remaining salts and small molecules in the gel pieces may contaminate the eluted peptides, and the additional water interferes with the preparations on the sample plate for MALDI-TOF analysis. Separation of the process into two plates eliminated this problem.

An important issue concerning automation is robustness of the assay. In particular, direct elution of the peptides onto the MALDI target is a critical step. Reliability of this process is mostly hampered by the inevitable presence of gel pieces in the in-gel digest, because gel pieces tend to block capillaries especially at moderate pressures. Due to the proposed two-plate principle, the processing of gel pieces was separated from the sample cleanup and spotting part; therefore, small volumes of elution solution were reliably dispensed into the wells on top of the capillaries. In this way, the gel pieces could not interfere with the dispensing of the

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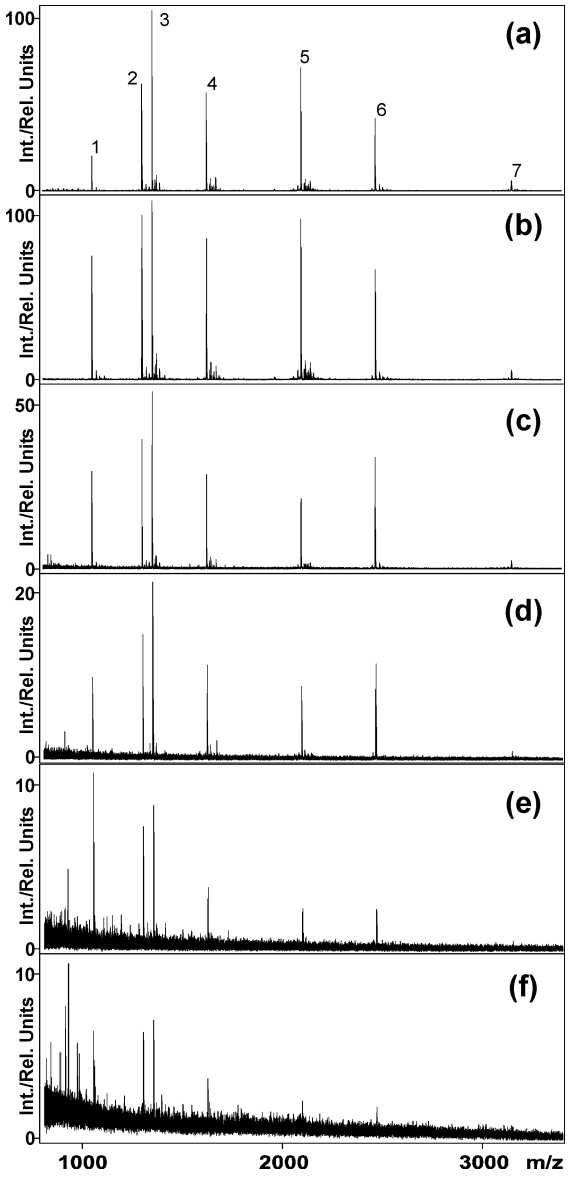

Figure 2. MALDI-TOF spectra of a standard peptide mixture: 1, human angiotensin II; 2, angiotensin I; 3, substance P; 4, bombesin; 5, ACTH clip 1−17; 6, ACTH clip 18−39; 7, somatostatin 28, showing the recovery from the SPE plate. Amounts of (a) 500 fmol, (b) 100 fmol, (c) 10 fmol, (d) 1 fmol, (e) 500 amol, and (f) 100 amol per peptide were bound to the solid phase, washed, and eluted directly onto a 600-μm AnchorChip target.

elution solution or block the capillaries to lower the efficiency of the elution.

The performance of the SPE plate separated from the rest of the assay was assessed using a simple peptide mixture of known composition. Different amounts of peptides in the same volumes as used in the in-gel digestion process were bound to the solid phase. In this way the in-gel digestion process could be mimicked and the exact amounts of peptides were known. Subsequently, the peptides were washed and eluted directly onto a 600-μm AnchorChip MALDI-TOF sample plate. The mass spectra representing the different peptide concentrations are compared in Figure 2. Even at 100 amol, peptides were observed in the spectrum indicating efficient binding and recovery from the monolithic material. This is also illustrated by the small elution volume of 2 μL used, which was transferred through the capillary

Table 1. 1D Gel of BSA: Sequence Coverage and Number of Identified Peptides from In-Gel Digestion

BSA/fmol	sequence coverage/%	no. of peptides identified
4000	38	21
400	67	44
200	67	40
100	71	42
40	51	28
30	39	22
20	42	23

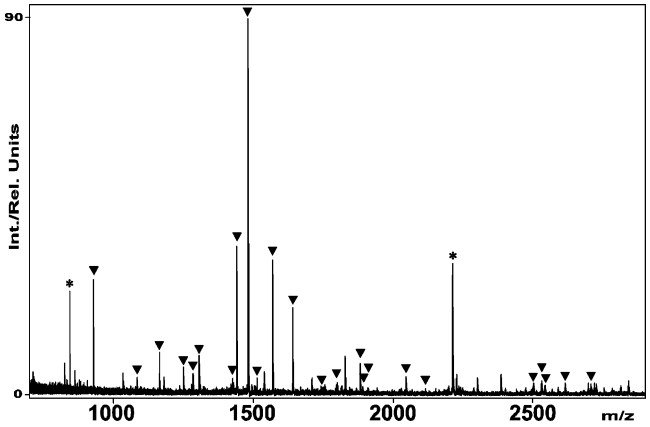

Figure 3. MALDI-TOF spectrum of tryptic in-gel digest of BSA from a coomassie-stained 1D gel. The gel piece contained 20 fmol of BSA. Peptide signals matched to BSA are marked with a triangle. Trypsin autoprotolysis products are indicated with asterisks.

by means of vacuum. No significant changes in relative intensities compared to a direct dried droplet preparation of the peptide mixture on the same sample support were observed (data not shown). A specific peptide preference in addition to hydrophobicity was therefore excluded.

To assess the utility of the proposed technology in terms of the whole in-gel digestion process, gel pieces from a coomassie-stained 1D gel with known amounts of BSA were processed according to the scheme in Figure 1. The gel bands at lowest concentrations were no longer visible and were therefore excised according to the known positions of the bands. The results of searching the obtained MALDI-TOF spectra in the SwissProt database are shown in Table 1. Above 400 fmol, a decrease of the sequence coverage could be observed, which was attributed to a saturation phenomenon of the solid phase in the capillaries. At 4 pmol of BSA, a sequence coverage of 38% was obtained, being sufficient for confident identification. The mass spectrum of the processed 20-fmol BSA sample is shown in Figure 3. The high signal-to-noise ratio in the spectrum and a sequence coverage of 42% ensured reliable identification. The results show a high dynamic range of the system, allowing proteins of faint coomassie spots to be identified successfully.

The compatibility of the presented system with silver staining techniques was illustrated by the in-gel digestion of human serotransferrin from a silver-stained 1D gel. A MALDI-TOF compatible staining technique according to Shevchenko et al.[2] was used. Samples were processed as described in Figure 1 with the exception that no destaining was carried out prior to digestion. MALDI-TOF analysis and database search yielded 50% sequence

Table 2. Identification of Yeast Proteins from Weak Spots on a Coomassie-Stained 2D Gel

protein identified	acc no.[a]	sequence coverage/%	CAI[b]
proteasome component Y7	P23639	31	0.14
phosphoribosylamidoimidazole-succinocarboxamide synthase	P27616	59	0.23
spermidine synthase	Q12074	59	0.36
SEC14 cytosolic factor	P24280	71	0.30
twinfilin A	P53250	46	0.14
protein phosphatases PP1 regulatory subunit SDS22 and ADP-ribosylation factor GTPase-activating protein GCS1	P36047 and P35197	55 and 68	0.18 and 0.15
glucokinase	P17709	55	0.16
probable ATP-dependent RNA helicase SUB2	Q07478	58	0.37
transcriptional modulator WTM1	Q12363	47	0.25

[a] Accession numbers correspond to the SwissProt database. [b] Codon adaptation index (see ref 33).

Table 3. SYPRO Ruby-Stained 2D Gel: Identification of Proteins from Liver Extract

spot no.[a]	protein identified	acc no.[b]	sequence coverage/%	score[c]	no. of MS/MS exp[d]
1	aldehyde dehydrogenase	P11884	43	158	0
2	protein disulfide isomerase A3	P11598	46	159	0
3	*S*-adenosylmethionine synthetase	P13444	31	78	0
4	prohibitin	P24142	75	275	0
5	3-hydroxyanthranilate 3,4-dioxygenase	P46953	44	152	0
6	thioredoxin-dependent peroxide reductase	P20108	32	170	2
7	proteasome subunit β type 4	P34067	37	145	2
8	guanine nucleotide-binding protein	P04901	25	98	1
9	ornithine carbamoyltransferase	P00481	34	205	1
10	3-α-hydroxysteroid dehydrogenase	*P*23457	42	103	1
11	serum amyloid P-component	*P*23680	15	121	1
12	F-actin capping protein	P47754	58	130	0
13	annexin V	P14668	57	208	2
14	LMW phosphotyrosine protein phosphatase	P41498	59	204	2
15	biliverdin reductase A	P46844	18	53	1

[a] Numbers refer to the gel picture shown in Figure 4. [b] Accession numbers correspond to the SwissProt database. [c] Probability score calculated by the Mascot software. [d] MS/MS and peptide mass fingerprint data was combined for the database search using the Biotools software.

coverage (data not shown) from a processed gel piece containing 50 fmol of transferrin. Transferrin is a highly stable protein containing a large network of disulfide bonds. Efficient reduction and alkylation of the resulting cysteine residues is therefore mandatory for high sequence coverage.

The presented method was applied to proteins from yeast extract separated on a coomassie-stained 2D gel. Weakly stained spots were excised (gel not shown) and processed in a fully automated fashion on a robotic workstation according to Figure 1, omitting reduction and alkylation (see Experimental Section). The identified proteins are listed in Table 2. High sequence coverage ranging from 31 to 71% was observed, providing unambiguous identification. In a faint spot, a mixture of two proteins was identified using the latest Mascot software version 1.9. The codon adaptation index[33,34] (CAI) is included in Table 2. CAI is a measure of gene expression levels and consequently a rough estimate of protein abundance. A value close to 1 means highly abundant protein whereas close to 0 is low abundant. A number of low-abundant proteins with CAI in the range of 0.1−0.2 were identified in the yeast extract (Table 2).

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SYPRO Ruby-stained gels present a particular challenge to the in-gel digestion system. The used fluorescent dye is not visible by eye, and a spot-cutting device with a fluorescent imaging system is necessary to pick the selected spots. The staining technique is compatible with MALDI-TOF, and SYPRO Ruby-stained gel pieces can be treated in the same way as coomassie-stained spots. However, SYPRO Ruby is a highly sensitive dye allowing the visualization of extremely low abundant proteins. Consequently, faint spots on a SYPRO Ruby-stained 2D gel can be used as an excellent measure of the sensitivity of the in-gel digestion system.

Spots of strong, medium, and weak intensities (Table 3) were picked arbitrarily from SYPRO Ruby-stained gel of a liver extract (Figure 4) to probe dynamic range and sensitivity of the system. Digestion and analysis were performed as described for the coomassie-stained spots with the exception that in the case of medium- and weak-intensity spots, MS/MS spectra were accumulated to improve confidence of identification. Proteins from intensively stained spots were identified, which means that the SPE plate has sufficient capacity for purifying and concentrating peptides in an in-gel digestion process. Proteins from barely visible spots on the SYPRO Ruby gel were identified as well (Table 3),

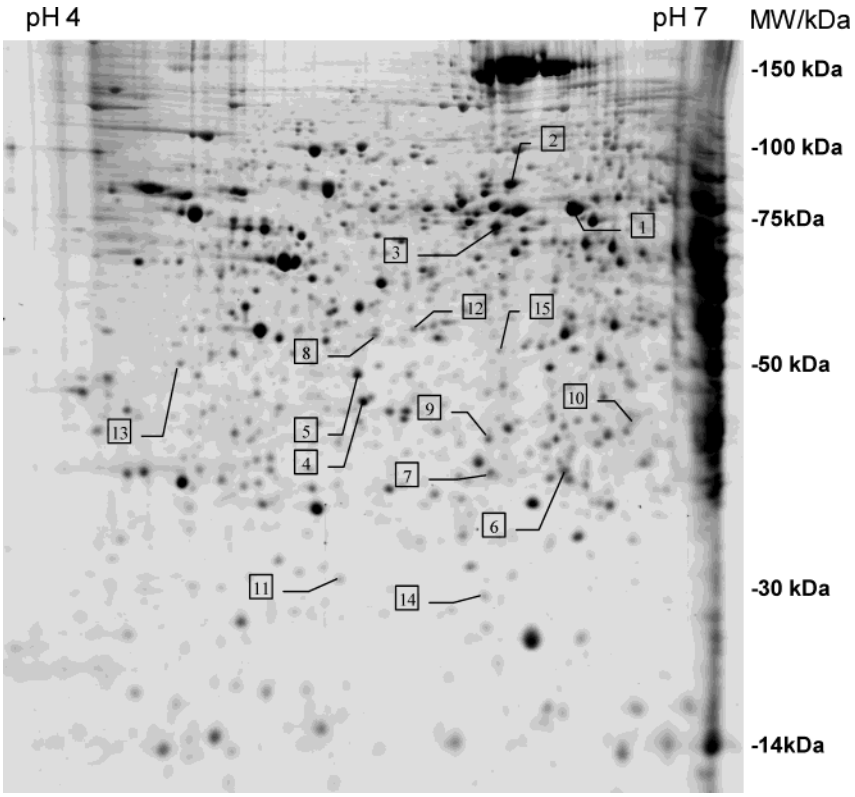

Figure 4. SYPRO Ruby-stained 2D gel of a liver extract. The processed spots are marked on the gel. The numbers refer to the list in Table 3.

proving the method to be compatible with the most sensitive staining techniques.

CONCLUSIONS

The described method for in-gel digestion of proteins in 1D and 2D gels facilitates parallel preparation and analysis of large numbers of samples. It applies to most staining techniques available to date and is sensitive enough to allow identification of the lowest abundant proteins on 2D gels. Work is ongoing in our laboratory to modify a robotics platform to integrate direct transfer of plates from an automated spot cutter to the workstation as well as transfer of the MALDI-TOF sample plate to the mass spectrometer. Sample plates will be processed in a highly parallel fashion and estimated maximum throughput will be 3000 samples/24 h. The current development will be applied to large-scale proteome studies undertaken by our laboratory.

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