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Matrix-assisted laser desorption–ionization mass spectrometry peptide mass fingerprinting for proteome analysis: identification efficiency after on-blot or in-gel digestion with and without desalting procedures

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Abstract

In theory, peptide mass fingerprinting by matrix assisted laser desorption–ionization mass spectrometry (MALDI-MS) has the potential to identify all of the proteins detected by silver staining on gels. In practice, if the genome of the organism investigated is completely sequenced, using current techniques, all proteins stained by Coomassie Brilliant Blue can be identified. This loss of identification sensitivity of ten to hundred-fold is caused by loss of peptides by surface contacts. Therefore, we performed digestion and transfer of peptides in the lower μl range and reduced the number of steps. The peptide mix obtained from in-gel or on-blot digestion was analyzed directly after digestion or after concentration on POROS R2 beads. Eight protein spots of a 2-DE gel from *Mycobacterium bovis* BCG were identified using these four preparation procedures for MALDI-MS. Overall, on-blot digestion was as effective as in-gel digestion. Whereas higher signal intensities resulted after concentration, hydrophilic peptides are better detected by direct measurement of the peptide mix without POROS R2 concentration.

Keywords: Peptide mass fingerprinting; Proteome analysis; Proteins; Peptides

1. Introduction

Proteome analysis needs high-resolution techniques adequate for the complexity of the biological material to be analyzed [1]. Despite the fact that several chromatography, electrophoresis and mass spectrometry methods are combined for proteome approaches [2–4], two-dimensional electrophoresis (2-DE) remains today the method with the highest resolution, up to 10 000 protein species may be separated on a single gel [5]. Peptide mass fingerprinting (PMF) by matrix-assisted laser desorption–ionization mass spectrometry (MALDI-MS) [6–10] is the method of choice for large-scale identification of proteins from 2-DE gels. The sensitivity of the identification of gel-separated proteins is several orders of magnitude below the sensitivity of MAL-

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DI-MS peptide measurements; therefore desalting and concentration procedures have been introduced. Concentration methods include: a peptide-collecting device with C_{18} reversed-phase material [11], peptide adsorption on reversed-phase chromatographic beads [12,13], ZipTips (Millipore) and microcolumns [14]. Salts can be removed after sample application to the MALDI-MS template by washing directly on the template, if the thin-layer [15], or the seed-layer technique [16] is used. For salt removal, non-porous polyurethane membranes as a sample support were introduced to allow washing [17]. Thin layer, seed-layer and two-layer [18] method improved the homogeneity of the sample surface, an important prerequisite for automatic MALDI-MS. Elution of the peptide mix from a nano-column by 50–100 nl matrix directly onto the MALDI-MS target [19] and further miniaturization has led to a microchip immobilized enzyme reactor [20]. A principally different technique is the molecular scanner [21,22]. All proteins of a 2-DE gel are first simultaneously digested during electrotransfer onto a PVDF membrane. The membrane is then directly scanned by MALDI-MS. Reduction of the volumes from the 100–500 μl range to the 5–10 μl range and the reduction of the ionic strength in the digestion buffer to 2.5 m*M* [23] increased the sensitivity of identification clearly. Using nitrocellulose in sample preparation for MALDI-MS led to increased yields of peptide $[M+H]^+$ ions [24]. If peptides are added directly to a sample of nitrocellulose dissolved in acetone, a 10-fold enhancement to the detection of tryptic peptide fragments was observed [25]. In another investigation proteins were blotted onto nitrocellulose membranes, stained with Ponceau S, digested by CNBr and the resulting peptide mix was analyzed after dissolving the membrane in matrix solution [26].

Here we present a comparison of on-blot with in-gel digestion procedures either by direct measurement or after concentrating on POROS R2 beads. The identification efficiency measured by the sequence coverage was not influenced by the preparation method used. Hydrophilic peptides were better detected by direct measurements. For automation the direct methods have an advantage because of simplicity without loss of quality.

2. Experimental

2.1. 2-DE and blotting

Cell proteins of *Mycobacterium bovis* BCG Chicago were prepared as described [27]. 2-DE gels combining non-equilibrium pH gradient gel electrophoresis with anodic sample preparation and a final gel size of 23 cm×30 cm×1.5 mm [5] resulted in the separation of about 1800 protein spots [27]. Four gels were produced with equal protein amounts (160 μg). Two gels were stained with Coomassie Brilliant Blue (CBB) R-250 [28]. The other two gels were used for semi-dry blotting [29] onto nitrocellulose membrane (Protran BA83, pore size 0.2 μm, Schleicher and Schüll, Dassel, Germany). Proteins were stained on the membranes for 5 min in a staining solution containing 25% isopropanol, 10% acetic acid and 0.1% Amidoblack. The background staining was removed with a destaining solution containing 25% isopropanol and 10% acetic acid by shaking two-times for 5 min each. Before drying the blots were washed with distilled water. Eight different protein spots were cut out from the 2-DE gels or membranes for the comparison of identification efficiency.

2.2. Digestion by trypsin

The proteins were digested on-blot or in-gel in 10 μl or 20 μl of 50 m*M* ammonium bicarbonate buffer pH 7.8, 10%(v/v) acetonitrile. The digestion mix contained for on-blot or in-gel digestion either 0.05 μg or 0.1 μg trypsin (Promega, Madison, WI). The proteins were digested overnight at 37°C with shaking. Only one spot was used per digestion. An overview of the four different methods to prepare the spots for MALDI-MS is given in Fig. 1.

2.3. Method A: POROS desalting after on-blot digestion

After digestion the sample was centrifuged, the supernatant was removed but not discarded, and the membrane was dissolved in 10 μl acetone. Subsequently, 20 μg POROS R2 beads (Perseptive Biosystems, Framingham, MA) in 10 μl methanol

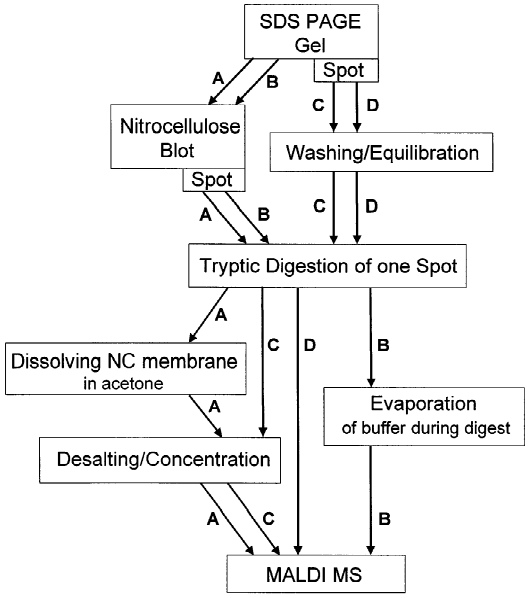

Fig. 1. Overview of the four different digestion and sample handling procedures compared in this investigation.

were added. The sample was dried together with the supernatant in a Speed Vac Concentrator (Eppendorf, Hamburg, Germany). For MALDI-MS, 5 μl matrix solution were added to the dried sample, sonicated for 2 min and then 2 μl were applied to the MALDI sample plate. The matrix solution used for all MALDI-MS measurements in this investigation was a saturated solution of α-cyano-4-hydroxy cinnamic acid (20 mg/ml) in 50% acetonitrile, 0.3% TFA.

2.4. Method B: on-blot digestion – direct measurement

During digestion the sample tubes were left open, resulting in evaporation of buffer. For MALDI-MS, 5 μl matrix solution were added to the dried sample, sonicated for 2 min and then 2 μl were applied to the MALDI sample plate.

2.5. Method C: in-gel digestion with poros desalting

Before digestion the spot was washed and equilibrated [11]. The digestion buffer was used as equilibration buffer. The digestion was performed in 20 μl digestion buffer with 0.1 μg trypsin as described above. After digestion the sample was centrifuged and sonicated for 2 min. Then, 10 μg POROS R2 beads in 100 μl 0.5% methanol, 0.1% TFA were added. After incubation for 15 min under shaking the POROS beads were centrifuged and transferred onto the sample plate. On-target elution was performed with 1 μl matrix solution [12].

2.6. Method D: in-gel digestion – direct measurement

For this method 2 μl of the sample described in method C were taken off directly after sonication of the digest, mixed with 2 μl matrix solution and 2 μl were applied onto the sample plate.

2.7. MALDI-MS

The mass spectra were obtained by a Voyager Elite MALDI-TOF mass spectrometer (Perseptive Biosystems). All measurements were performed in the positive-ion reflector mode at an accelerating voltage of 20 kV, 70% grid voltage, 0.05% guide wire voltage and a delay of 100 ns. 256 scans were averaged per spectrum. The low-mass gate was set at 500 m/z.

2.8. Bioinformatics

The standard gel of cell proteins from *Mycobacterium bovis* BCG Chicago was evaluated using the program Topspot (Algorithmus, Berlin, Germany). Spot positions and intensities were determined. Proteins were identified after PMF using the search program MS-Fit (http://falcon.ludwig.ucl.ac.uk/ucsfhtml3.2/msfit.htm). The following search parameters were applied: The sequence database of NCBI was reduced to the mycobacterial proteins. A mass tolerance of 0.1 Da and two incomplete cleavages were allowed. The protein molecular mass was reduced to ±20% of the 2-DE determined molecular mass. Acetylation of the N-terminus, alkylation of cysteine by acrylamide, removal of methionine from the N-terminus of the protein and concurrent acetyla-

tion, and pyroGlu formation of N-terminal Glu were considered.

The high-performance liquid chromatography (HPLC) index, as a parameter for the elution behavior and hydrophobicity of the peptides, was determined with the protein analysis program GPMAW (Lighthouse data, Odense, Denmark). The HPLC index calculation based on a C_{18} reversed-phase system with separation in TFA/acetonitrile [30].

3. Results

3.1. Spot characteristics

The 2-DE pattern of *Mycobacterium tuberculosis* proteins resolved about 1800 protein species, from which eight single separated spots with randomly distributed molecular mass and isoelectric point and of different staining intensities were selected (Table 1). One gel spot or membrane spot was used per digestion. The spot numbers in Fig. 2 correspond to the entries in the 2-D PAGE Database (http://www.mpiib-berlin.mpg.de/2D-PAGE) [27]. The Coomassie Brilliant Blue R-250 stained pattern (Fig. 2A) containing 160 μg protein shows a clear reduction of resolution as compared to the silver stained pattern (Fig. 2B) containing 60 μg protein. The number of protein species resolved on CBB gels may be increased by applying up to 900 μg protein and staining with CBB-G250. The eight spots selected randomly for this investigation appeared on the 2-DE pattern well visible even with 160 μg protein applied to the gels and the less sensitive CBB R-250 staining.

3.2. MALDI-MS fingerprints

The aim of the investigation was to find a procedure for protein digestion and peptide transfer with optimal sequence coverage for identification. Technical restrictions relating to the digestion methods used in this study made it necessary to apply different percentages of the protein spot applied to the mass spectrometer. For both on-blot digestion procedures 40% of the sample was applied. A minimum of 5 μl had to be used to cover all the beads and the membrane. In all experiments 2 μl were applied to the template. For the direct measurement from the in-gel digest only 5% can be applied because for the gel spot size used here at least 20 μl digestion buffer is necessary to cover the gel. We did not want to dry the sample to avoid additional loss of peptides. Subsequently, 2 μl were mixed with 2 μl matrix and 2 μl were applied to the template. The complete POROS beads were applied after the in-gel digestion and therefore 90% were analyzed by MALDI-MS.

For some of the samples produced by direct procedures, the crystallization of matrix appeared not to be uniform on the sample plate. This problem could be overcome by redissolving the sample on the sample plate in 1 μl of additional matrix solution. In general it was observed that the crystallization of the desalted samples was more uniform. The peptide mass fingerprint contained nearly the same mass peaks independent of the preparation method. As an example the peptide mass fingerprints of spot C272 are shown in Fig. 3. The intensity of the peaks

Table 1
Characterization of the protein spots investigated[a]

Spot	p*I*	M_r	Intensity	Protein name
A382	5.36	54265	273	Succinate-semialdehyde dehydrogenase
C171	5.71	29258	447	35 kDa antigen
C191	4.73	28160	385	Electron transfer flavoprotein b subunit
C272	5.12	17353	108	Single strand binding protein
C653	5.26	29779	393	Steroid dehydrogenase
D91	6.51	18781	200	Member of AhpC/TSA family
E160	5.51	15312	357	Conserved hypothetical
F52	8.01	10935	468	30S ribosomal protein S6

[a] Spot numbers refer to the numbers in the 2D-PAGE database (http://www.mpiib-berlin.mpg.de/2D-PAGE) [27].

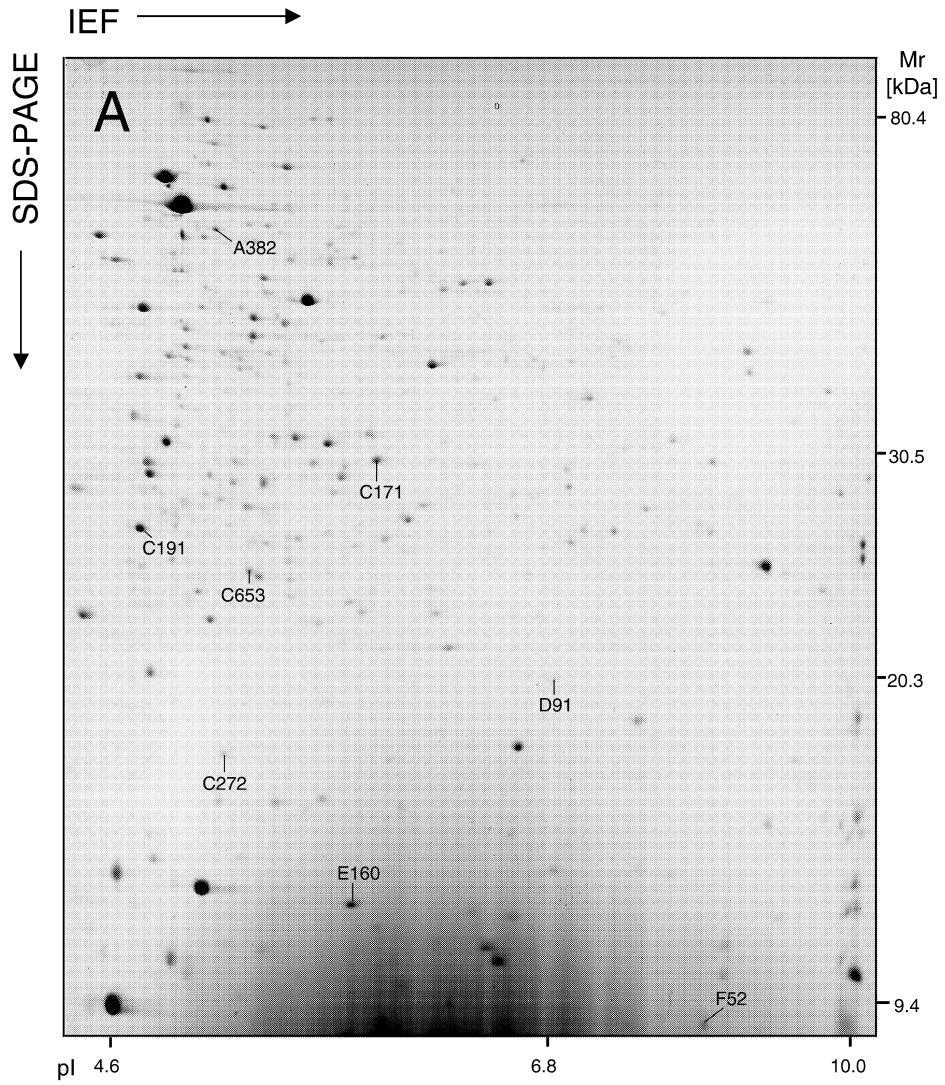

Fig. 2. 2-DE pattern of *Mycobacterium bovis* BCG Chicago cell proteins. The protein species used as test proteins in this investigation were marked with accession numbers of 2D-PAGE database. (A) 160 μg protein were separated by 2-DE and stained with CBB-R250. (B) 60 μg protein were applied to 2-DE and stained with silver.

observed was within the same range for the on-blot methods. The intensity was decreased as compared with those of the in-gel digestions. Measurement of POROS concentrated sample after in-gel digestion resulted in an increased peak intensity for peptides with a M_r>1500 and a decreased number of peptides with a M_r<1500. In the case of on-blot digestion, one additional peptide (M_r 618.298) was detected by direct measurement.

3.3. Hydrophobicity of the peptides

The calculation of the HPLC-index of the additional peptides in the direct methods revealed that some of the most hydrophilic peptides were lost in the POROS procedures (Fig. 4). This effect was also found for the other seven proteins (Table 2). Whereas the number of matched peptides with an HPLC index below 15 (hydrophilic peptides) was always

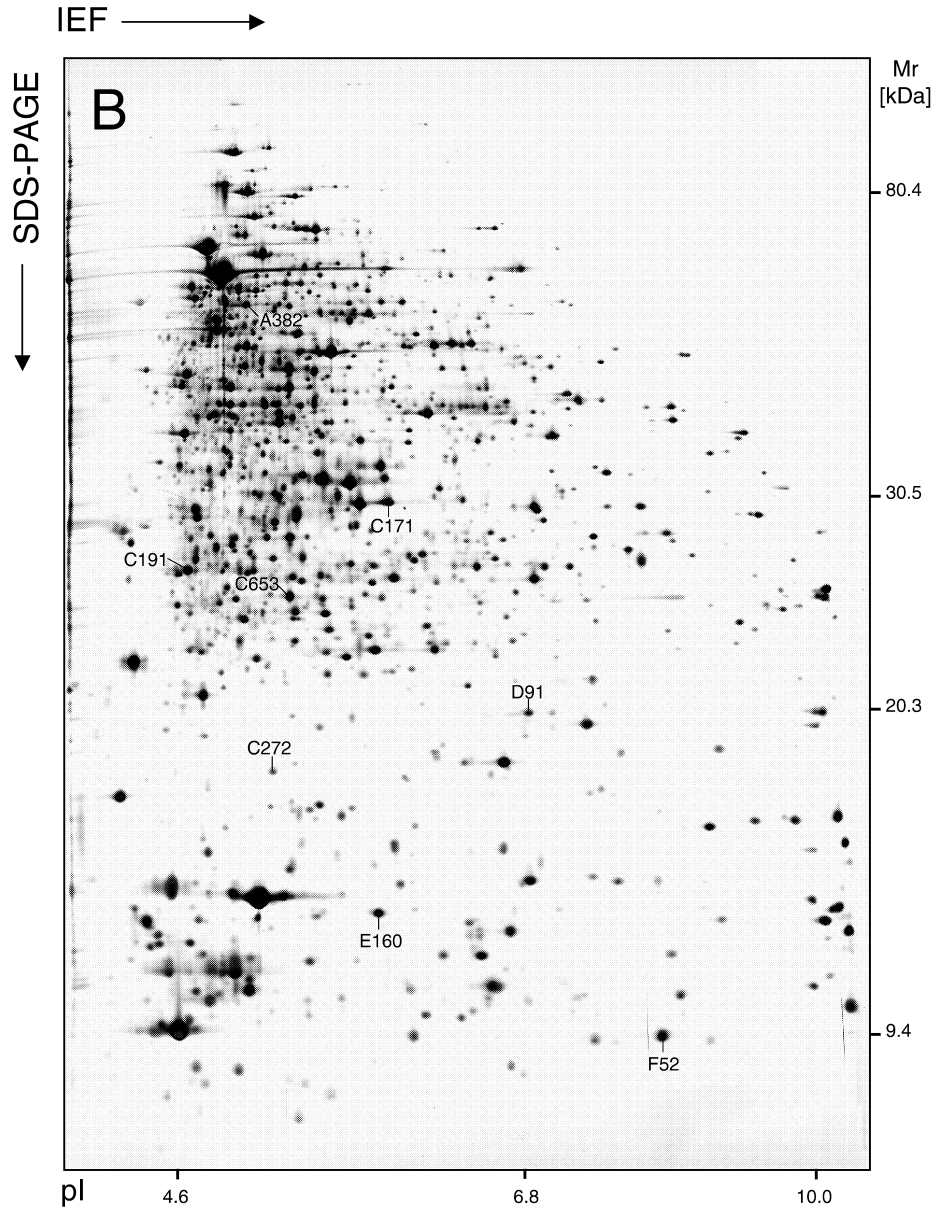

Fig. 2. (*continued*)

lower with POROS concentrated samples, the number of matched peptides with an HPLC index higher than 15 was nearly the same in all cases. In addition, for the hydrophobic peptides the percentage of the total intensity was higher for six of the eight proteins when POROS was used for concentration. Regarding the POROS procedure, on average, 86% of the total intensity of the matched peptides was found in an HPLC index range over 15 (Table 2). Only 14% of the total intensity were found in the HPLC index range below 15. Using the direct measurement procedure we found a shift of the intensities to the lower HPLC index range, and 33% of the total intensity were found in this range.

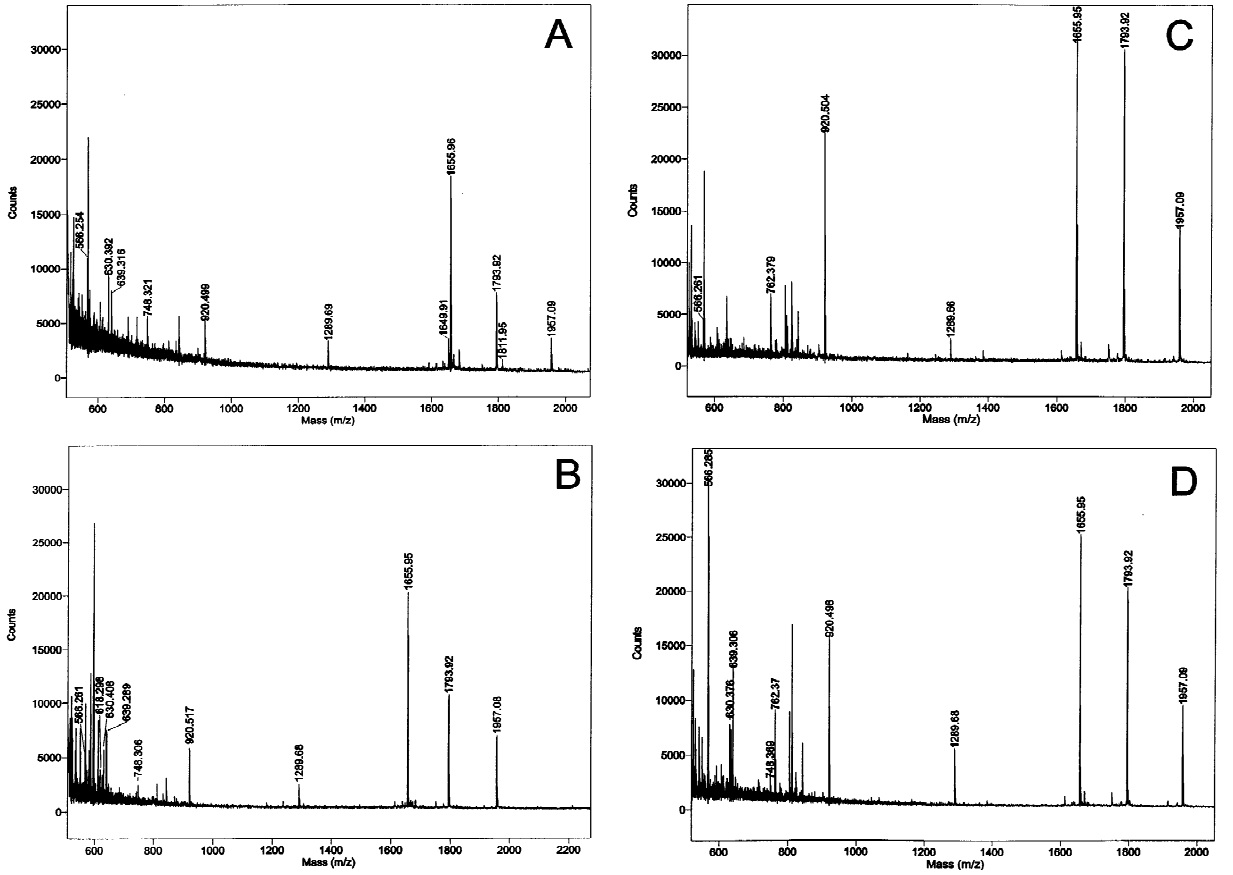

Fig. 3. MALDI-MS fingerprints of Spot C272 after using different digestion procedures. (A) On-blot digestion, POROS concentration, (B) on-blot digestion, direct measurement, (C) in-gel digestion, POROS concentration, (D) in-gel digestion, direct measurement. Numbers at the top of the peaks mark all matching peptides.

3.4. Sequence coverage and number of matched peptides

The sequence coverage describes how many amino acids of a protein sequence are covered by the peptides found in the peptide mass fingerprint experiment. In our experience, 30% sequence coverage of a protein is sufficient for identification [27]. The sequence coverage, therefore is a quality criterion for the sample preparation. Nearly all methods resulted in sufficient sequence coverage for identification of all proteins investigated (Fig. 5). Only in one case the 30% sequence coverage criterion was not reached (C171, 28%). The sequence coverage depends more on the protein investigated than on the procedure used for digestion and transfer to MS.

Another criterion for the quality of a peptide mass fingerprint is the number of matched peptides. For comparison of direct and POROS concentrated measurement, we investigated in-gel digestion procedures in detail (Fig. 6). The total number of matched peptides and the number of peptides in different HPLC index ranges were compared. For five of eight proteins the total number of matches was increased using the direct measurement, twice the number was equal and once the number was higher using the POROS concentration method. The number of peptides with an HPLC-Index <15 was higher using the direct method for six of eight proteins and for the remaining two proteins it was equal. On average there were two additional matching peptides found with the direct procedure (Table 2). The effect of

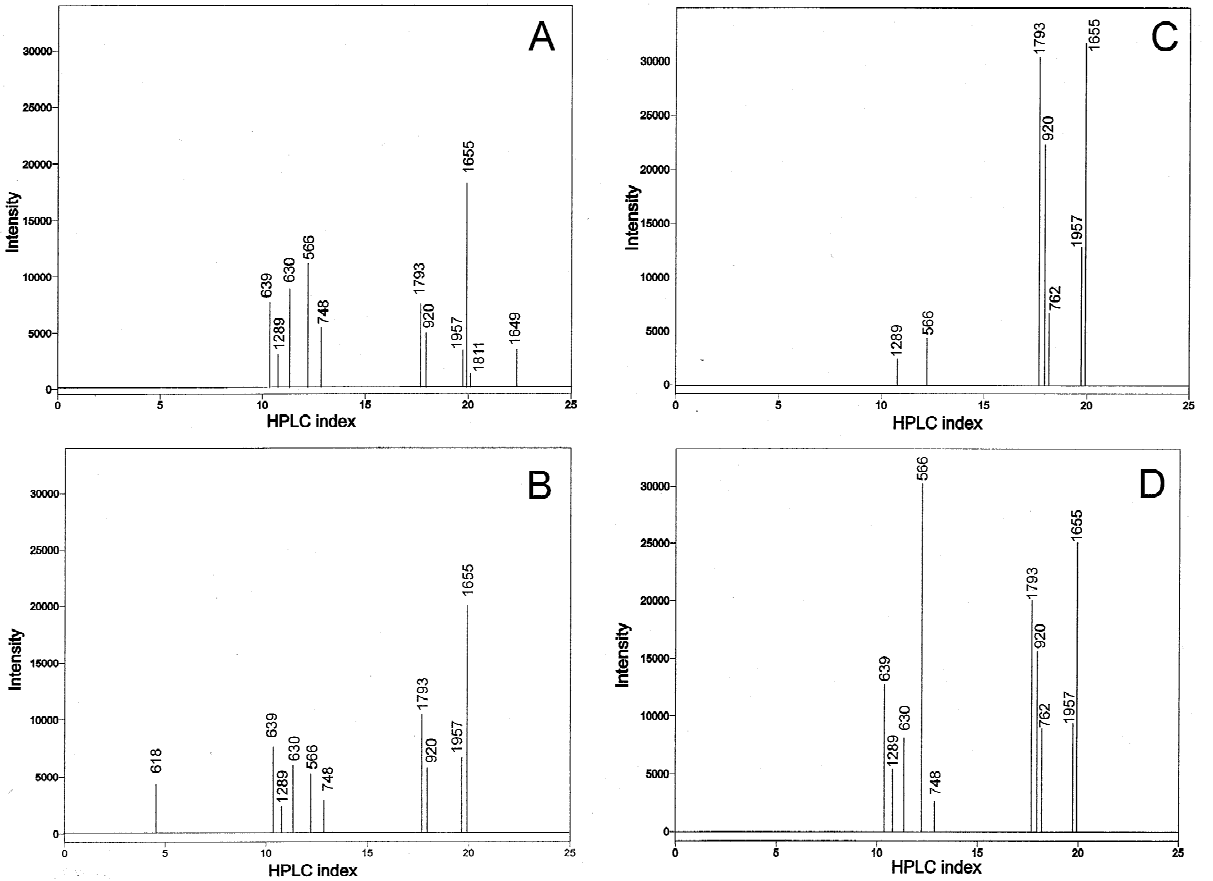

Fig. 4. Intensity and HPLC index of the peaks in the mass spectra of Spot C272. The numbers indicate the masses of the peptides. (A) On-blot digestion, POROS concentration, (B) on-blot digestion, direct measurement, (C) in-gel digestion, POROS concentration, (D) in-gel digestion, direct measurement.

Table 2
In-gel digest procedure: percentage of matched peptides relative to total intensity, number of matched peptides regarding their HPLC index range, and total number of matched peptides

Spot No	HPLC index≤15				HPLC index >15					
	% of total intensity		No. of matched peptides		% of total intensity		No. of matched peptides		Total no. of matched peptides	
	Direct	POROS	Direct	POROS	Direct	POROS	Direct	POROS	Direct	POROS
A382	82	38	11	7	18	62	13	13	24	20
C171	35	6	5	2	65	94	9	7	14	9
C191	42	33	5	4	58	67	10	11	15	15
C272	43	6	5	2	57	94	5	5	10	7
C653	27	3	4	2	73	97	13	12	17	14
D91	0	0	0	0	100	100	6	8	6	8
E160	23	24	3	2	77	76	4	4	7	6
F52	10	3	1	1	90	97	6	6	7	7
Ø	33	14	4.25	2.5	67	86	8.25	8.25	12.5	10.75

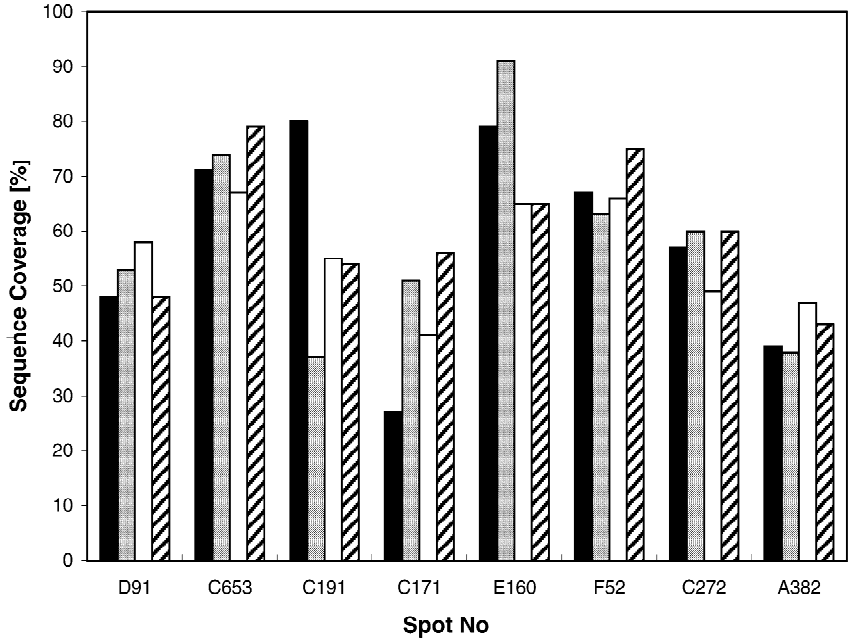

Fig. 5. Comparison of sequence coverage obtained after MALDI-MS PMF of peptides after various digestion procedures. Black columns, on-blot digestion, POROS concentration; gray columns, on-blot digestion, direct measurement; white columns, in-gel digestion, POROS concentration; hatched columns, in-gel digestion, direct measurement.

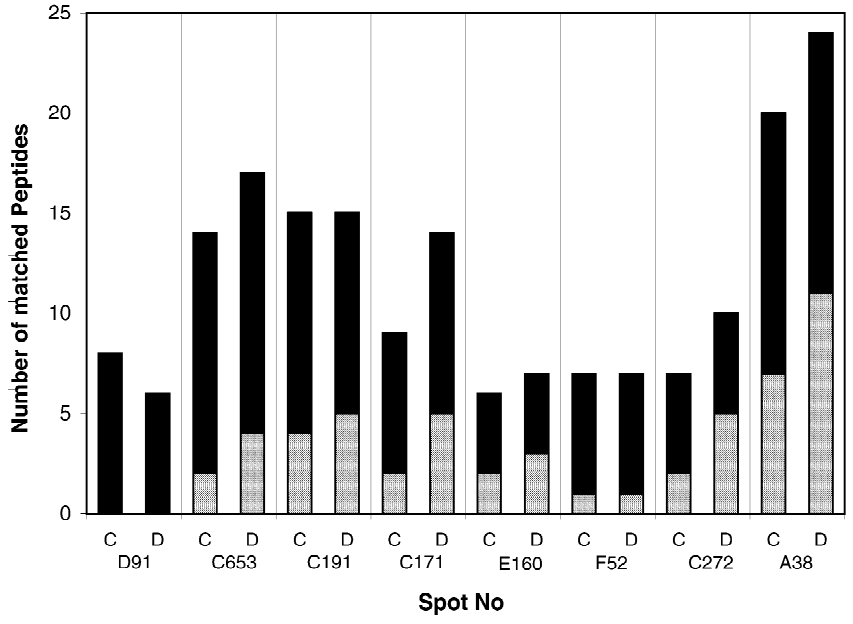

Fig. 6. Comparison of the number of matched peptides with regard to their HPLC index. C, in-gel digestion, POROS concentration, and D, in-gel digestion, direct measurement. Black part of the columns: HPLC-index>15, gray part of the columns: HPLC-index<15.

preference for hydrophobic peptides in the POROS procedures was confirmed by an evaluation of the most intense peak per spectrum. The most intense peak in the spectra of the direct gel procedure is found with four of eight peptides in a lower or with four of eight peptides the same HPLC index than the most intense peak of the spectra of the POROS procedure.

3.5. Sensitivity

A final statement of the most sensitive method cannot be given because the same percentage of the protein spot was not used for all experiments. For the two on-blot procedures the same amount was applied and nearly the same intensities were found on the mass spectra (Fig. 3). In contrast, the intensities detected for in-gel digests were about the same as those for mass spectra for both POROS and direct measurements, despite the fact that only 5% were applied in the direct measurement as compared with 90% in the POROS procedure. This result demonstrates the potential of the direct measurements.

4. Discussion

4.1. Necessity of improving the identification efficiency

Two highly sensitive methods are often combined in proteome analysis: 2-DE and MALDI-MS. The detection limit of spots on 2-DE patterns depends on the detection method. Using CBB, 1 to 10 pmol of protein can be detected within a 2-DE gel. Silver staining [31–33], negative staining [34] and fluorescence staining [35] reveal 10–100 fmol of protein. For radiolabelled proteins the detection limit is at least three orders of magnitude lower [36].

Sensitivity in the amol range has been described for peptides in MALDI-MS. Using the Voyager Elite MALDI mass spectrometer we recorded detection limits in the range of 10–100 fmol for several synthetic peptides, if 2 μl of sample–matrix mixture were applied to the MS-template as described in Experimental section. The sensitivity of the identification by PMF is limited by several factors. In the case of silver staining, the staining procedure produces a reduction of peptides by redox reactions resulting in a sequence coverage, which is too low for identification [37]. CBB G-250 staining [38] results in alkylation of acidic amino acids [39] leading to additional mass peaks. Furthermore, using conventional techniques the transfer of the peptides to the MALDI-mass spectrometer causes loss of peptides, not allowing the identification of proteins by PMF below the CBB level. We therefore analyzed two factors in detail: the starting point, spots from gels or blots; and the treatment of the peptide mixture, the effect of including a concentrating step.

4.2. On-blot vs. in-gel digestions

In comparison with a previously published digestion procedure [11] we exchanged the Tris buffer with a volatile buffer and reduced both the buffer volume and trypsin concentration. This resulted in successful identification of single spots. For on-blot digestion addition of Zwittergent 3-16 [40] or octyl-beta-D glucopyranoside [11] was not necessary, because the protein was digested on the membrane, which was then dissolved either in acetone or directly in the matrix releasing the peptides. The recovery of peptides from membrane after digestion and elution with detergents was with 70% not quantitative [40]. Dissolving of the membrane in organic solvent is one possibility to overcome this problem and the use of nitrocellulose membranes (NC) makes it possible to recover large peptides and intact proteins [26]. Although NC was used as an additive in MALDI-MS [23–25], it is difficult to handle, because the final NC concentration in the matrix solution should not exceed 3 mm^2/20 μl [26]. The combination of dissolving of the membrane with direct measurement of the resulting solution or with concentration of peptides by using reversed-phase material [12] resulted in identification efficiencies, which were comparable with those for in-gel digestions. The on-blot procedure may result in a better protein to gel or membrane relationship, which overcomes the disadvantage of potential loss of protein during blotting. Using the described modifications, both in-gel and on-blot digestion procedures resulted in comparable sequence coverage. For automation, the on-blot direct measurement would be most advantageous, because it is the simplest pro-

cedure containing only two steps. The fact that the same identification efficiency was obtained for the direct measurement of in-gel digests whilst using only about 5% of the sample size used after the POROS concentrating procedure, shows that there is further potential in increasing the sensitivity in the direct in-gel digestion methods.

4.3. Direct vs. poros-concentrated sample preparation

Standard protocols for concentration recommend starting with several protein spots, digesting with Tris buffer, working with volumes of 100–500 μl, and concentrating and washing of the peptide mix on reversed-phase material [11]. We developed a more direct protocol and after starting with one spot, digestion in ammonium carbonate buffer, and working with volumes of 10–50 μl, we obtained improved identification efficiency without washing and concentration of the peptide mix. In our experiments there was a shift to more hydrophilic peptides in the mass spectra of the directly analyzed peptides. The loss of hydrophilic peptides during desalting with POROS material could be due to the inefficient binding of these peptides to the reversed-phase material. An important factor is the percentage of solvent used in the digestion and in our experiments the solvent percentage was about 2%. It has been previously reported [12] that the final solvent concentration should not exceed 2.5%. A higher percentage could have a negative effect on the adsorption efficiencies. Obviously, even 2% solvent reduces the portion of the hydrophilic peptides.

Direct measurements using a low-salt buffer system have also been described by Fountoulakis et al. [23]. In contrast to their results, we succeeded with both methods (direct and POROS concentrated). Fountoulakis et al. used higher volumes for the concentrating procedure, which may have resulted in a loss of peptides. The POROS concentrated samples resulted in higher intensity in the mass spectra. Some advantages of storage for POROS bound peptides have also been described [13], and we found that storage of the peptide mixture without POROS addition revealed poor mass spectra (data not shown).

Despite the fact that we found one or two additional peptides by using direct measurements, giving more certainty in the database search, higher sequence coverage was not observed. In some cases we obtained an increase in the sequence coverage by combining the results of the direct measurement and the POROS desalted measurement. The direct procedures have the major advantages that they are simpler and require less time, an important prerequisite for automation. The measurements were performed directly after digestion without time-consuming concentration steps. A disadvantage was the uneven crystallization of sample and matrix on the MALDI target. It was observed that it is more difficult to find ‘hot spots’: regions on the target where peak intensity is high. This could be due to some interference of the buffer or other contents of the digest. A prerequisite for post-source decay (PSD) sequencing is sufficient peak intensity. Therefore, the fact that the hydrophilic peptides are present with high intensity makes it possible to investigate these peptides with PSD.

5. Conclusion

We tested a number of methods for peptide digestion and analysis and found equivalent sequence coverage for all methods, some procedures however are advantageous for particular applications. This shows a remarkable robustness for the peptide mass fingerprints. The peptide mass fingerprint is characteristic for a protein, and is largely independent from the preparation method. It is therefore helpful to introduce peptide mass fingerprints into 2-DE databases and use them for identification of proteins avoiding the sequence database search. A prerequisite for successful automation of digestion is the use of procedures with a low number of steps. The on-blot procedure without desalting required only application of digest buffer and dissolving of peptides in matrix and is preferable for automatic procedures. The direct in-gel digestion method shows the potential of further increase of sensitivity, an important prerequisite to profit from the high sensitivity of fluorescence and radioactivity detection on 2-DE gels. The POROS in-gel digestion method leads to enough sample volume for further characterization such as sequencing by PSD or MS/MS.

6. Nomenclature

MALDI-MS	matrix-assisted laser desorption–ionization mass spectrometry
2-DE	two-dimensional electrophoresis
PMF	peptide mass fingerprinting
CBB	Coomassie Brilliant Blue

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