

High Sensitivity Identification of Membrane Proteins by MALDI TOF-MASS Spectrometry Using Polystyrene Beads

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Abstract: Membrane proteins play a large variety of functions in life and represent 30% of all genomes sequenced. Due to their hydrophobic nature, they are tightly bound to their biological membrane, and detergents are always required to extract and isolate them before identification by mass spectrometry (MS). The latter, however remains difficult. Peptide mass fingerprinting methods using techniques such as MALDI-TOF MS, for example, have become an important analytical tool in the identification of proteins. However, PMF of membrane proteins is a real challenge for at least three reasons. First, membrane proteins are naturally present at low levels; second, most of the detergents strongly inhibit proteases and have deleterious effects on MALDI spectra; and third, despite the presence of detergent, membrane proteins are unstable and often aggregate. We took the mitochondrial uncoupling protein 1 (UCP1) as a model and showed that differential acetonitrile extraction of tryptic peptides combined with the use of polystyrene Bio-Beads triggered high resolution of the MALDI-TOF identification of mitochondrial membrane proteins solubilized either with Triton-X100 or CHAPS detergents.

Keywords: mass spectrometry • detergent • membrane protein identification • peptide mass fingerprinting

Introduction

Genome-wide analysis of integral membrane proteins showed that 30% of open reading frames encode membrane proteins containing one to twenty membrane spans, with an overrepresentation of 6 and 7 transmembrane spans proteins in bacterial and human genomes, respectively.¹ In the plasma

membrane, at the interface between the external environment and the inside of the cell, membrane proteins are essential for osmotic homeostasis, substrate transport, and cell signaling. Intracellular organelles also contain a large family of membrane proteins. For instance, the human anion carrier family from the inner membrane of the mitochondria consists of up to 75 members.² All of them possess a specific sequence signature (PROSITE accession number PS00215), share a threefold internal sequence repeat,³ and transport anionic substrates of mitochondrial metabolism both to and from mitochondria.⁴ The best known of these carriers is the ADP/ATP carrier (ADT1), whose atomic structure has been recently solved by 3-D crystallization of the protein.⁵

Peptide mass fingerprint (PMF) is a protein identification method based on the specificity of a mass spectrum of the peptide mixture resulting from the digestion of a protein by an enzyme. This method is dependent on the number of peptides generated and/or detected for a given protein, which defines its sequence coverage. Known atomic structures of membrane proteins as well as statistical analysis of their topology has revealed that, in eukaryotic genomes, membrane spans are very often connected by short loops.¹ Consequently, identification of membrane proteins by PMF is limited by the lack of hydrophilic domains that are accessible to proteases and by the presence of detergent. To reach reasonable sequence coverage and significant identification score, it is therefore necessary to solve the problem of the detection of transmembrane peptides by MS analysis.⁶ To address this question, we have chosen the uncoupling protein 1, a member of the mitochondrial carrier family related to ADT1, which is specifically and highly expressed in brown adipocyte mitochondria,⁷ as a membrane protein model for MALDI-TOF MS analysis. Among the three methods of in-gel tryptic digestion of the protein that were tested, one of them increased the sequence coverage of UCP1 protein by including most of its transmembrane domains. We also postulated that traces of detergent tightly bound to membrane proteins may decrease the efficiency of tryptic peptide recovery and consequently the MS sensitivity for membrane protein identification. To test our hypothesis, polystyrene beads were used to remove the detergent from the gel band containing the protein prior MS analysis. Polystyrene beads significantly improve the PMF

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identification of mitochondrial membrane proteins solubilized either in Triton X-100 or CHAPS detergent.

Given that transmembrane peptide detection represents the limiting aspect of membrane protein identification by MS, and furthermore that the MALDI-TOF MS approach is becoming a more common and "user-friendly" tool for biologists, our results may be useful for membrane proteome research in the field of cell biology and medicine.

Experimental Procedures

All reagents necessary for sample preparation, gel electrophoresis, and MS were of the highest grade available from Sigma (Saint Quentin Fallavier, France). Sequencing grade modified trypsin was purchased from Promega (Charbonnières, France). CHCA matrix was obtained from Bruker Daltonics (Wissenbourg, France).

Purification of Native UCP1. Mice were cold acclimated for a week's time at 4 °C following the guideline of the Comité Régional d'Éthique pour l'Expérimentation animale Ile de France-René Descartes (agreement number P2.BM.004.03.). Brown adipose tissue (BAT) mitochondria were purified as previously described,⁸ and UCP1 protein was purified on hydroxyapatite column as initially shown by Lin and Klingenberg⁹ and following the protocol described previously.¹⁰ After separation on 12% acrylamide/bis-acrylamide SDS-PAGE, proteins were silver stained following the method of Shevchenko and colleagues.¹¹

In-Gel Tryptic Digestion. Protein bands were excised from 1-D SDS-PAGE, transferred into a tube containing 1% acetic acid in water, and the piece of gel was destained with the Invitrogen silver staining kit following the manufacturer's instructions.

Method A. Gel pieces were submitted to four washing steps with a thermomixer comfort high-speed rocking (Eppendorf, Maryland). First, the gel was washed twice for 20 min with 25 mM ammonium bicarbonate solution. Second, the gel was rinsed for 15 min with 25 mM ammonium carbonate in 50% acetonitrile. Third, the gel was placed for 15 min in 25 mM ammonium bicarbonate. Finally, the gel was washed with water. Gels pieces were dehydrated in the DNA mini speed vacuum rotor (Heto, Victoria, Australia) and hydrated for 30 min on ice with a solution of 25 mM ammonium bicarbonate and 5 mM CaCl₂ solution containing trypsin (12 ng/ μ L). After overnight digestion at 37 °C with trypsin, the supernatant was removed and kept in a separate tube. Peptides were further extracted from the gel with 0.1% trifluoroacetic acid (TFA) in water and pure acetonitrile. All supernatants were pooled and dried out in a speed vacuum rotor. The pellet was resuspended in 15 μ L of 0.1% TFA in water, and the solution was sonified for 5 min in the Branson 5510 water bath sonifier (Branson, California). Tryptic peptides were further purified by C18 ZIP-TIP chromatography. After washing the ZIP-TIP with 100% acetonitrile and with 0.1% TFA, the sample was loaded and the contaminants were eluted with 0.1% TFA. Tryptic peptides were sequentially eluted with 4 μ L of 50 and 80% acetonitrile in water.

Method B. The piece of gel was washed for 10 min with 200 μ L of milli-Q water, washed for 10 min with 100 μ L of 12.5 mM ammonium bicarbonate in 50% acetonitrile, and finally dehydrated with 100% acetonitrile. For the cysteine carbamidomethylation, dried gel was placed at 56 °C for 45 min in a reducing solution containing 10 mM DTT and 25 mM ammonium bicarbonate. The supernatant was removed, and

alkylation of the cysteines was achieved by incubation of the gel for 45 min in the dark with 55 mM iodoacetamide in 25 mM ammonium bicarbonate buffer. Gel pieces were washed with 25 mM ammonium bicarbonate in 50% acetonitrile and subsequently dehydrated with 100% acetonitrile. Tryptic digestion was performed as described above for method A. Extraction of tryptic peptides was achieved by successive incubation of the gel with 35 μ L of 1% TFA in 50% acetonitrile and with 35 μ L of pure acetonitrile. For each extraction, samples were sonified for 10 min in a waterbath sonifier. Fractions were pooled and dehydrated with a speed vacuum rotor. The pellet was resuspended in 5 μ L of 0.1% TFA in 50% acetonitrile and further sonified for 15 min in a waterbath sonifier.

Method C. This method was adapted from method A and B. Samples were washed following method B, and after tryptic digestion, peptides were extracted according to method A.

Polystyrene Beads Treatment. Bio-Beads SM2 (20–50 mesh) polystyrene beads were purchased from Bio-Rad (Marnes la Coquette, France). The gel protein band was first divided into two equal pieces. About 15 mg of Bio-Beads, pretreated with methanol and washed three times with milli-Q water, were incorporated to the sample at the first washing step with 200 μ L of milli-Q water. After 1 h incubation with the Bio-Beads at room temperature, the piece of gel was taken out, introduced in a new tube, and further processed according to method B. Methods A, B, and C are schematically shown in Figure 1.

MALDI-MS Analysis. Saturated alpha-cyano-4-hydroxycinnamic acid (α -CHCA) matrix was prepared by incubating about 10 mg of matrix with 100 μ L of 0.1% TFA in 50% acetonitrile. The mixture was sonified for 5 min in a waterbath sonifier and centrifuged for 5 min at 14 000 rpm. Fifty microliters of supernatant was ready to use after dilution in 100 μ L of 0.1% TFA in 50% acetonitrile. The sample (0.5 μ L) was spotted on a steel MALDI target plate, 0.5 μ L of freshly made CHCA matrix was added to the sample, and the mixture was left to dry at room temperature. Peptides were analyzed by MALDI-TOF MS using an Autoflex instrument (Bruker Daltonics, Wissenbourg, France) exactly as described by Brouillard et al.¹² SWISSPROT was chosen as database for protein identification.

ESI-MS/MS Analysis. After MALDI-MS analysis, the remaining tryptic peptides were analyzed by LC-MS/MS. The system consisted of a nanoflow liquid chromatography system (Ultimate 2000, Dionex) coupled to an ion trap mass spectrometer (HCT plus, Bruker Daltonics). Peptides were separated by reversed phase HPLC on a C18 Pepmap 100 column (LC packings) with a 60 min gradient of 3–90% acetonitrile in 0.1% formic acid at a flow rate of 300 nL/min. The column eluent was sprayed directly into the mass spectrometer, and the instrument was operated in positive mode. Data-dependent MS/MS spectra were acquired automatically and then searched against the NCBI non-redundant database using Mascot software (Matrix science).

Results

UCP1 was selected as a membrane protein model to assess the efficiency of two protocols that are widely used for the in-gel tryptic digestion of protein prior to MS analysis. BAT mitochondria were solubilized in 5% Triton X-100, and UCP1 was purified on hydroxyapatite column with a final concentration of Triton X-100 estimated around 2%. After silver staining of the SDS-PAGE gel, the UCP1 band was excised and differentially processed following methods A and B (Figure 1). The two methods differ at the washing step 2 and at the peptide

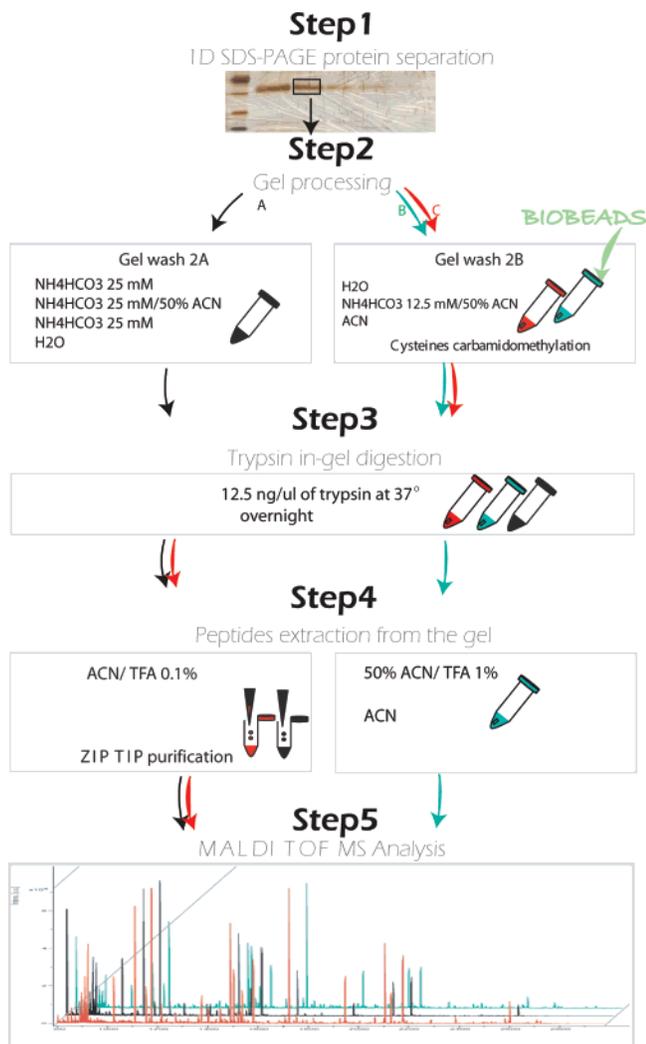


Figure 1. Simplified representation of the in-gel tryptic digestion by methods A, B, and C. Method A: The gel was washed with 25 mM ammonium bicarbonate, 25 mM ammonium carbonate in 50% acetonitrile, and water. After tryptic digestion, the gel was washed with a solution of 0.1% TFA in 50% acetonitrile, and peptides were collected using a C18 phase ZIP-TIP column. Method B: Washing of the acrylamide gel was performed with water, with 12.5 mM ammonium carbonate in 50% acetonitrile, with 100% acetonitrile, and was followed by the carbamidomethylation of cysteines residues. Extraction of tryptic peptides was achieved by simply washing the gel with 1% TFA in 50% acetonitrile and with pure acetonitrile. Method C was made with step 2 of method B and step 4 of method A. Bio-Beads, when used, were added in the first wash of method B as indicated by the arrow. All samples were subjected to MALDI-TOF analysis using CHCA as matrix.

extraction step 4 (Figure 1 and see Experimental Procedures). Figure 2 shows the MALDI-TOF MS spectra obtained with both methods. In the mass detection range $800 < m/z < 1700$, spectra were not significantly different. However, above $1700 m/z$, many more peaks of high intensity were present in the spectrum obtained with method B. These peaks were attributed to UCP1 peptides (Figure 2B, stars). Method B revealed 19 UCP1 peptides covering up to 69% of its sequence whereas method A identified 10 peptides only covering 33% of UCP1 sequence (Figure 2 and Table 1). To understand why method B was more efficient than method A for UCP1, we designed a hybrid method, C, comprised of step 2 from method B and step

4 from method A (Figure 1). A mass spectrum of intermediate quality was obtained with method C (Figure 2C), leading to the identification of UCP1 with a score of 84, which is to be compared with a score of 155 for method B and 50 for method A (Table 1). The most remarkable feature of method B is that 12 hydrophobic transmembrane peptides of UCP1 were identified whereas only 7 were identified with method A and 8 with method C (Table 1). Method B was also more suitable for the detection of high m/z value peptides. At higher masses ($m/z > 1700$), methods A, B, and C allowed for the identification of 2, 9, and 4 peptides, respectively. The sensitivity of method B was then tested using an example of both a membrane protein (UCP1) and a soluble protein (BSA). Serial 2-fold dilutions of UCP1 proteins were performed in purification buffer supplemented with 2% Triton X-100. BSA was simply diluted in water without detergent. All samples were run on SDS-PAGE, gels were silver stained (Figure 3, A and B), and samples were processed according to method B and subjected to MS. The UCP1 identification score dramatically decreased below 40 pmoles of UCP1 protein (Figure 3A), whereas 300 fmoles of BSA were sufficient to identify the BSA with an acceptable score (Figure 3B). Despite the improvement achieved in the sensitivity of membrane protein identification, this remains 100-fold lower compared to the sensitivity of soluble protein identification. We then postulated that tightly bound detergent molecules interfere with the detection of low amounts of a membrane protein by MS. It is known that many detergents inhibit the cocrystallization of tryptic peptides with the matrix which, in turn, decreases the efficiency of peptide ionization and the intensity coverage of the spectrum. To test our hypothesis, polystyrene beads were inserted at various steps of method B to remove the Triton X-100 present in UCP1 protein samples. Incubation of the sample with Bio-Beads before SDS-PAGE did not improve the quality of the MS spectrum (data not shown), probably because the ratio of detergent to Bio-Beads was too high. Incubation of Bio-Beads with purified tryptic peptides just before mixing the solution with the CHCA matrix completely suppressed the MS spectrum, suggesting that, in this condition, Bio-Beads had not only trapped the detergent but also most of the peptides. To overcome these difficulties, Bio-Beads (15 mg) were added to 200 μ L of water in the first wash of step 2, before the tryptic digestion (Figure 1). After 1 h of incubation, the piece of acrylamide gel was transferred into a new tube and processed following method B. Bio-Beads treatment had no effect on the sequence coverage at 200 pmol of UCP1, whereas the same treatment significantly increased the sequence coverage of UCP1 at lower concentrations (Table 2). [We cannot give an estimate for each protein band because they overlap on the gel (see Figure 5, BAT fraction). However, knowing that total protein amount loaded on the gel was 100 pmol, and the fact that UCP1 protein is highly expressed in BAT, it can be assumed that UCP1 amount is within the range of 60–80 pmol.] Furthermore, the treatment improved tenfold the detection limit of UCP1 protein, up to 4 pmoles (Figure 4A). They also improved the reproducibility of the method (Figure 4B). To further demonstrate that Bio-Beads are of general interest for PMF of membrane proteins, mitochondrial carrier proteins other than UCP1 were identified. Mitochondrial carriers are differentially expressed in different tissues, and several of them can be purified on a hydroxyapatite column in a manner similar to UCP1. Therefore, kidney, heart, liver, and BAT mitochondria were solubilized with Triton X-100, and mitochondrial carriers were purified on hydroxyapatite column.

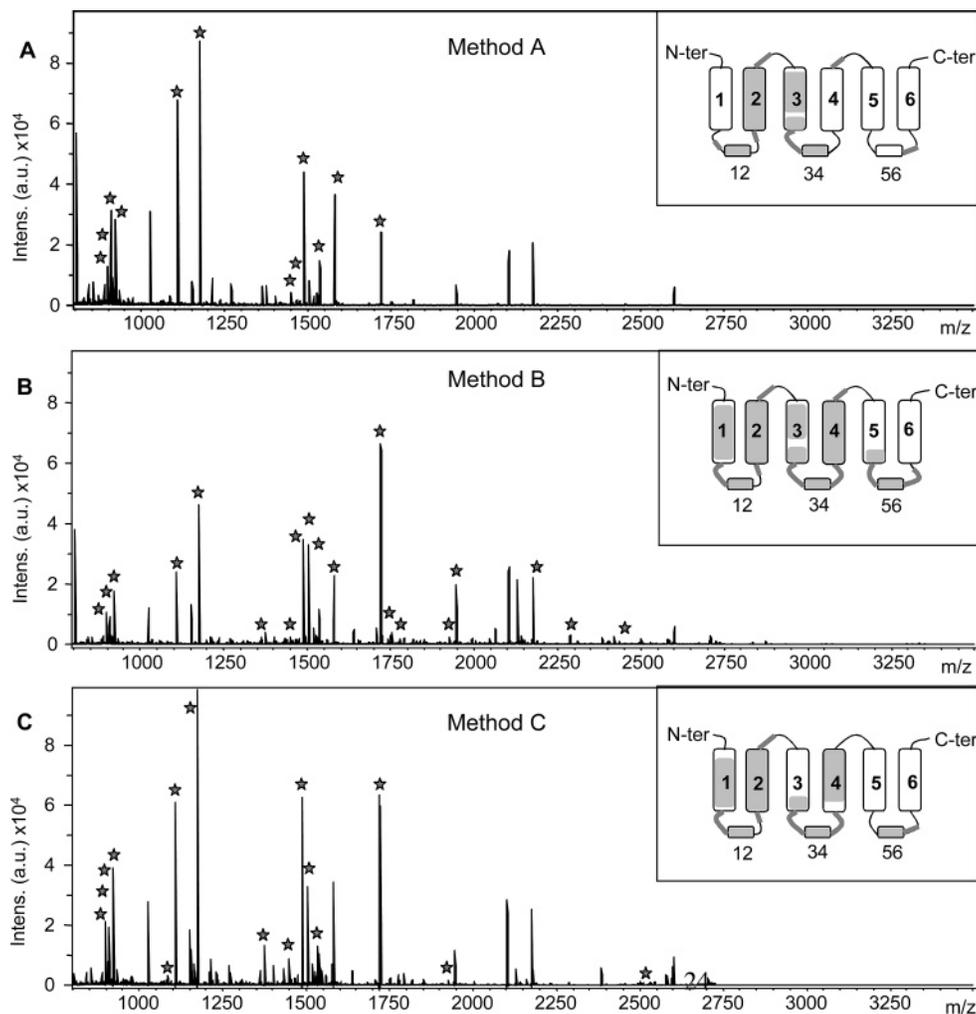


Figure 2. Representative MALDI-TOF spectra of the purified UCP1 protein. After hydroxyapatite purification of UCP1, 200 pmole of protein were loaded on SDS-PAGE and subjected to in-gel digestion with trypsin following methods A, B, and C, as indicated. Mass spectra were obtained on a Bruker Autoflex instrument; processed automatically by FlexAnalysis, Biotoools, and Mascot software using the same parameters. Stars highlight peaks ascribed to UCP1 tryptic peptides. On the right corner of each spectrum is drawn the simplified topology of UCP1 protein according to Miroux et al.³² Helices are numbered according to the structure of the ADP/ATP carrier.⁵ Shaded areas indicate the sequence covered by the identified UCP1 tryptic peptides.

Table 1. MALDI-TOF MS Identification of UCP1 According to Methods A, B, and C^a

PMF of UCP1 (200 pmole)	A	B	C
Mascot score	50 ± 7	155 ± 37	84 ± 6
sequence coverage (%)	33 ± 7	69 ± 3	47 ± 8
number of peptides	10 ± 3	19 ± 0.6	15 ± 3
number of membrane peptides	7 ± 0.3	12 ± .4	8 ± 0.2
peptides with 800 < <i>m/z</i> < 1200	6 ± 0.3	5 ± 0.3	7 ± 0
peptides with 1200 < <i>m/z</i> < 1700	4 ± 0.3	5 ± 0	5 ± 0
peptides with 1700 < <i>m/z</i> < 2200	2 ± 0.3	5 ± 0	3 ± 0.7
peptides with 2000 < <i>m/z</i> < 3300	0 ± 0	4 ± 0.3	1 ± 0.3

^a All values represent the mean of three independent experiments.

Flow-through fractions were loaded onto SDS-PAGE gel (Figure 5), and after silver staining of the gel, samples from 10 protein bands were cut, treated according to method B, with and without addition of Bio-Beads, and were subjected to MS analysis. Table 2 summarizes all membrane proteins identified from the four tissues. In most cases, the use of Bio-Beads increased the identification score and the sequence coverage of the membrane protein identified. For instance, MCAT identification score was 41 without Bio-Beads and increased

to 74 after Bio-Beads treatment. Similarly, Bio-Beads treatment increased by 50% the identification score of three proteins identified from heart mitochondria: the ADP/ATP translocase 1, the 2-oxoglutarate/malate carrier, and the phosphate carrier protein. These proteins were present as a mixture in the same gel band (band 4). More importantly, three proteins could not be detected at all without the Bio-Beads treatment: VDAC1 from BAT and kidney mitochondria (Table 2, bands 10 and 2, respectively) and acylcarnitine carrier (MCAT) from liver (band 8). To extend the usefulness of Bio-Beads to the removal of detergent other than Triton-X100, heart mitochondrial proteins were solubilized with 4% CHAPS, and 10 μ g of proteins were loaded and separated on a 1-D SDS-PAGE. Proteins of molecular weight comprised between 30 and 45 kDa were systematically identified by PMF MS analysis (Table 3). Once again, Bio-Beads improved the identification score or the sequence coverage of almost all proteins identified. For example, the score of ETFA, a mitochondrial flavoprotein, increased from 44 to 81 and the sequence coverage of the protein increased from 50 to 63%. Moreover, MPCP and M2OM mitochondrial

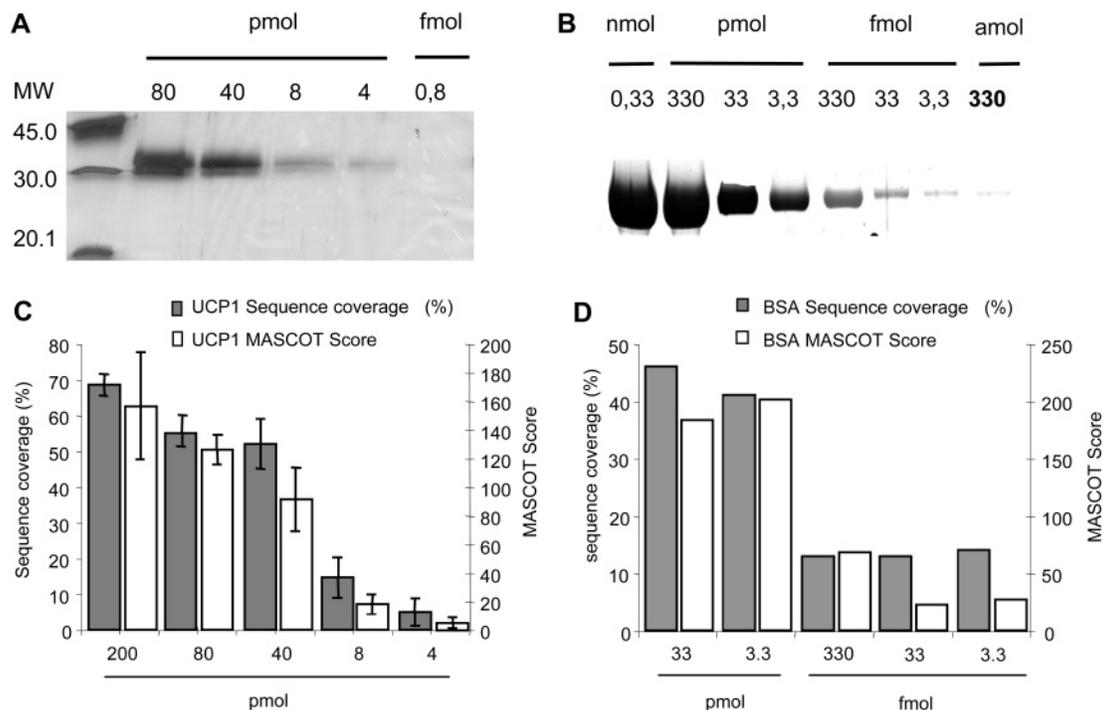


Figure 3. Sensitivity of the in-gel tryptic digestion by method B. Serial dilutions of UCP1 (A) and BSA (B) proteins were loaded onto SDS-PAGE. After silver staining of the gel, protein samples were excised, subjected to in-gel tryptic digestion following method B, and analyzed by MALDI-MS. Sequence coverage and Mascot identification score of UCP1 and BSA are represented in (C) and (D), respectively.

Table 2. Effect of Bio-Beads on PMF Identification of Mitochondrial Membrane Proteins after Purification on Hydroxyapatite

tissue (pmol of proteins ^a)	band	protein	accession numbers	MW	without Bio-Beads			with Bio-Beads		
					Mascot score	sequence coverage (%)	hit position	Mascot score	sequence coverage (%)	hit position
BAT (100)	10	UCP1	P12242	33095	39	31	>5	84	44	1
		MCAT	Q9Z2Z6	33005	41	35	>5	74	41	2
		VDAC1	Q60932	30737	not identified			43	43	5
heart (200)	4	ADT1	P48962	32752	92	40	1	140	56	1
		MPCP	Q8VEM8	39606	61	30	2	101	32	2
		M2OM	Q9CR62	34002	42	24	3	78	46	3
kidney (200)	3	MPCP	Q8VEM8	39606	47	23	1	66	24	1
		VDAC1	Q60932	30737	not identified			36	23	1
liver (200)	9	M2OM	Q9CR62	34002	30	23	>5	40	23	1
		ADT2	P51881	32779	25	23	>5	20	12	>5
	8	MCAT	Q9Z2Z6	33005	not identified			33	36	>5
		7	VDAC1	Q60932	30737	71	57	1	70	52
	MTCH2		Q791V5	33477	36	36	>5	39	28	3

^a Estimated from the Pierce BCA assay of the flowthrough fractions.

carriers could not be identified without Bio-Beads and were easily identified after Bio-Beads treatment.

To test compatibility with ESI-MS/MS analysis, UCP1 peptide samples prepared according to Method B were analyzed on nanoLC-MS/MS (HTCplus Bruker). Two UCP1 peptides, seven MCAT peptides, and two VDAC peptides were identified by MS/MS fragmentation (Table 4). This demonstrates that our method is compatible with ESI MS-MS identification and implies that the traces of detergent left did not preclude their identification. However, the Bio-Beads treatment did not improve the number of fragmented peptides. Altogether, a higher percentage of sequence coverage of mitochondrial membrane proteins was achieved using MALDI-TOF MS analysis of Bio-Beads-treated samples.

Discussion

The study of the membrane proteome is limited by several factors, i.e., solubilization, separation by bi-dimensional electrophoresis, and identification by MS. First, the distribution of charges in membrane protein sequences is not regular. Positively charged residues are enriched in the non-translocated part of the protein, following the so-called “positive-inside rule” that was first established by von Heijne for bacterial inner membrane proteins.¹³ Consequently, the number of tryptic peptides can be dramatically reduced. For instance, overnight digestion of the bacteriorhodopsin protein with trypsin generates only one peptide.¹⁴ To overcome this problem, cyanogen bromide has been used to cleave membrane hydrophobic peptides,¹⁵ alone or in combination with trypsin.¹⁶ Fischer and colleagues also showed that a combination of chymotrypsin

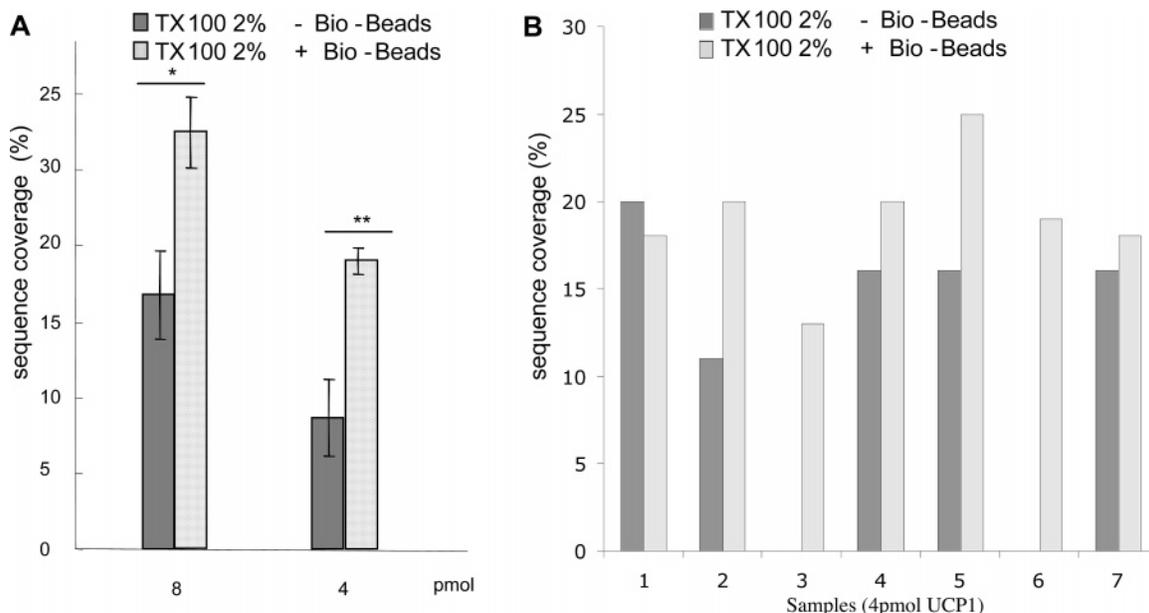


Figure 4. Bio-Beads increase the detection threshold of UCP1 and the reproducibility of method B. (A) UCP1 was diluted in 2% Triton X-100 to 4 and 8 pmol and analyzed by MS according to method B with or without Bio-Beads treatment. Stars indicate statistical significance in the Mann Whitney test: *, $P \leq 0.05$; **, $P \leq 0.001$. (B) Distribution of the sequence coverage values obtained with 4 pmol of UCP1 in 2% Triton X-100.

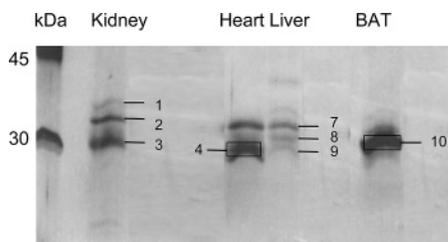


Figure 5. Purification of mitochondrial carriers other than UCP1. Mouse kidney, heart, liver, and BAT mitochondria were solubilized with Triton X-100, and mitochondrial carriers were purified on hydroxyapatite columns as described in Mozo et al.¹⁰ For each tissue, the flow-through fraction of the column was loaded on SDS-PAGE. Gel was silver stained and protein bands were numbered from 1 to 10 prior to MS analysis.

and staphylococcal peptidase I gave significantly better results than trypsin for the MPF analysis of bacteriorhodopsin.¹⁷ In contrast to bacteriorhodopsin, mitochondrial carriers do not seem to follow the positive-inside rule. For instance, tryptic digestion of UCP1 potentially generates 41 detectable peptides (one tryptic miscleavage and possible methionine oxidation allowed). In this work, we did not increase the number of tryptic peptides generated but rather the efficiency of tryptic peptide recovery. This is confirmed by the fact that the proportion of miscleaved peptides was around 20% in all three methods (not shown).

A second difficulty linked to membrane protein biochemistry is the difficulty to solubilize and to separate them on 2-dimensional gels (for review, see ref 18). Due to their high isoelectric point, they are badly separated by isoelectrofocusing (IEF) electrophoresis, and they also undergo aggregation at their isoelectric point. Alternative strategies have been developed to remove soluble proteins from membrane protein samples and to separate membrane protein complexes. Millar and Heazlewood carefully purified integral membrane protein from mitochondria by stripping mitochondrial membranes with so-

dium carbonate or by differential solubilization of the membrane proteins with a mixture of chloroform, methanol, and water.¹⁹ We and other laboratories have set up alternative separation systems based on the separation of membrane protein complexes on blue native gels instead of IEF gels^{12,20} or by using new classes of nonionic/zwitterionic detergents compatible with the first dimension of 2-D gel electrophoresis.²¹ In this paper, we have shown that 1-D SDS-PAGE of hydroxyapatite flow through is a reasonable alternative approach for the study of the sub-proteome of mitochondrial carriers. By doing so, we have identified mitochondrial carriers that were not found by Millar in plant mitochondrial membrane¹⁹ or by Lescuyer in the human mitochondrial proteome.²² Nevertheless, although prepurification of membrane or membrane proteins is useful, it is not sufficient for successful identification of membrane protein by PMF. The systematic comparison of sample preparation protocols carried out in this work showed that washing the gel prior to tryptic digestion as well as organic extraction of cleaved peptides are critical steps that lead to higher sensitivity and quality of MALDI-TOF spectra. In method B, washing the gel with 100% acetonitrile before tryptic digestion seems to help the final recovery of tryptic peptides. In addition, the use of C18 reverse phase ZIP-TIP led to a decrease in the m/z value of UCP1 peptides to below 1700 (Table 1).

However, the fact the BSA could be identified by PMF with much higher sensitivity than UCP1 prompted us to question the deleterious effect of the detergent used in the membrane protein sample preparation. The variable effect of detergents in the field of MS has long been a matter of debate and contradictory results. For instance, several laboratories have shown that SDS removal is necessary to allow the acquisition of MALDI-TOF MS spectra of soluble proteins²³ and to increase the sequence coverage of membrane proteins.²⁴ On the contrary, Breaux and colleagues²⁵ developed the concept of surfactant-aided MS analysis, showing that SDS had a positive effect on MALDI-MS detection of hydrophobic peptides without

Table 3. Effect of Bio-Beads on PMF Identification of Heart Mitochondrial Membrane Proteins Solubilized with CHAPS

band	name of the mouse identified protein	accession number	MW	without Bio-Beads		with Bio-Beads	
				Mascot score	sequence coverage (%)	Mascot score	sequence coverage (%)
1	ADT1, ADP/ATP translocase 1	P48962	32752	110	57	130	54
1	ADT2, ADP/ATP translocase 2	P51881	32779	49	37	61	36
2	MPCP phosphate carrier	Q8VEM8	39606	^a	^a	54	30
2	BDH, D-beta hydroxybutyrate dehydrogenase	Q80XN0	38260	33	35	42	32
2	ATPG, ATP synthase gamma chain	Q91VR2	32865	^a	^a	42	24
2	M2OM 2-oxoglutarate/malate carrier	Q9CR62	34002	^a	^a	40	26
3	PHB2 Prohibitin-2	O35129	33276	46	49	70	58
3	ETFA, electron transfert flavoprotein subunit alpha	Q99LC5	35018	44	50	81	63
3	HCDH, 3-hydroxy-CoA dehydrogenase	Q61425	34442	30	31	60	57
4	NDUA9, NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9	Q9DC69	42482	86	40	97	42
4	MDHM, Malate dehydrogenase	P08249	35574	85	53	105	53
4	ODPB, Pyruvate dehydrogenase E1 subunit beta	Q9D051	38912	40	34	45	38
5	THIM 3-ketoacyl-CoA thiolase	Q8BWT1	41831	53	45	44	48
5	AATM, Aspartate aminotransferase	P05202	47381	51	42	113	64

^a Not identified.

Table 4. ESI MS–MS Analysis of UCP1 Tryptic Peptides Prepared According to Method B

protein identification	number of peptides	sequence coverage (%)	Mascot score	<i>m/z</i>	peptide sequence	MS–MS fragmentation score
MCAT	8	19	344	907.76	EGITGLYR	45
				922.47	GFNAVMIR	40
				958.64	SVSDLSVPR	53
				1024.86	EEGVTSLYK	23
				1152.74	KLYQEFGR	56
				1227.99	ADEPKPISPFK	36
				1535.85	ELIREEGVTSLYK	51
UCP1	2	5	87	895.60	EGPTAFFK	33
				1107.61	YTGTYNAYR	55
VDAC1	2	8	45	1399.86	LTFDSSFSPNTGK	18
				1399.87	YQVDPDACFSAK	39

affecting the detection of hydrophilic peptides. Although the real effect of SDS detergent is still unclear, the concept of surfactant-aided MS analysis has been recently illustrated by the use of octyl beta glucopyranoside (OBG) detergent, which enhanced the digestion efficiency and the peptide recovery of several membrane proteins.^{16,26} This is in agreement with our observation that some detergent had a positive effect on MS analysis of membrane proteins. For example, bacterial recombinant UCP1 refolded with *n*-dodecyl phosphocholine detergent (Fos-choline-12, Anatrace) was identified by MALDI-TOF MS following method B with 70% sequence coverage. Unfortunately, native UCP1 as well as other mitochondrial carriers cannot be purified with the Fos-choline-12 detergent. Native UCP1 purified in Triton X-100 was more difficult to analyze by MS. Following method B, we obtained sequence coverage and an identification score (Table 1) higher than those previously published for UCP1²⁷ but lower than those obtained with the recombinant protein in Fos-choline-12 detergent. We therefore postulated that molecules of Triton X-100 remained tightly bound to UCP1 even after separation of proteins in SDS-PAGE which, in turn, inhibited the recovery of tryptic peptides. Addition of polystyrene beads to remove the detergent during sample preparation strongly improved the PMF identification of UCP1 and of several membrane proteins, mostly mitochondrial carriers, which co-purified in the flow through fraction of the hydroxyapatite column (Table 2). The usefulness of detergent removal by Bio-Beads for MS analysis was then extended to CHAPS detergent (Table 3). Polystyrene beads are widely used for the reconstitution of membrane proteins into

liposomes but also for trapping detergent during 2D and 3D crystallization.^{28–30} They nonspecifically bind hydrophobic organic molecules, preferentially detergents rather than lipids. Although polystyrene beads appear to work over a large spectrum of detergents, they seem to be most efficient at removing non-ionic detergents such as Triton X-100 and zwitterionic detergents such as CHAPS.³¹ We cannot exclude that polystyrene beads also remove hydrophobic contaminants other than just free detergent molecules, which in turn either facilitates the tryptic digestion of the protein and peptide recovery or simply reduced the MS background. Finally, because most of membrane proteins are expressed at low levels and require high amounts of detergent for their solubilization and stabilization, method B together with the Bio-Beads treatment of the sample may be extremely useful for structural analysis of membrane proteins by mass spectrometry.

Abbreviations

PMF, peptides mass fingerprinting; MALDI, matrix-assisted laser desorption/ionisation; TOF, time-of-flight; 1-D SDS-PAGE, one dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis; UCP, uncoupling protein; ADT, ADP/ATP translocase; BAT, brown adipose tissue; 2-D, two-dimensional; 3-D, three-dimensional; HTP, hydroxyapatite; TFA, trifluoroacetic acid; DTT, dithiothreitol; CHCA, alpha-cyano-4-hydroxycinnamic acid; MS, mass spectrometry; VDAC, voltage-dependent anion-selective channel protein; BSA, bovine serum albumine; PBS, phosphate-buffered saline; IEF, isoelectric focusing; OBG, octyl-β-glucopyranoside; Fc12, dodecylfoscho-

line; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; M2OM, mitochondrial 2-oxoglutarate/malate carrier protein; MPCP, phosphate carrier protein, mitochondrial; MTCH, mitochondrial carrier homolog; ACN, acetonitrile; a.u., arbitrary unit; MW, molecular weight.

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