A Simple and Effective Method to Analyze Membrane Proteins by SDS-PAGE and MALDI Mass Spectrometry

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Abstract. *Background/Aim: Identification and characterization of membrane proteins is a crucial challenge in proteomics research. Thus, we designed a novel method to prepare proteins possessing extensive hydrophobic stretches for mass spectrometry studies, without sacrificing other classes of proteins. Materials and Methods: This method uses sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) separation and relies solely on a matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) instrument, the most common and easiest to use mass spectrometer. Results: Using this analytical procedure, a significant number of hydrophobic peptides were recovered, with no reduction in overall sequence coverage and with a good identification of transmembrane proteins sequence. Applying this method to the systematic identification of proteins located in lipid rafts, up to 47% of identified proteins were obtained with an improvement of sequence coverage. Conclusion: The procedure presented here is suitable for both identifying purified hydrophobic proteins and systematically investigating hydrophobic protein mixtures. It can be easily applied even in non-dedicated laboratories, such as those mostly devoted to clinical chemistry.*

About 30% of all proteins in nature are either membrane associated (MA, mostly hydrophilic), or integral membrane proteins (IMP, mostly hydrophobic). Compared with MAs, IMPs are fully embedded into the phospholipid bilayer and play a cardinal role in cell-to-cell interactions, substrate transport, and signal transduction (1). Despite the great interest in characterizing membrane proteins, their strong hydrophobicity, mainly due to α-helical bundles, is a hurdle for most common proteomic approaches, such as sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), liquid chromatography, and mass spectrometry (2, 3). As a consequence, membrane proteins are usually poorly represented in proteomic analyses (4).

Purification of IMPs by gel electrophoresis presents a significant problem, since their hydrophobic nature makes them prone to precipitation, or to be trapped in the gel's mesh, mainly when the buffer pH reaches their isoelectric point, or in the absence of appropriate detergents and/or reducing agents. Since both trapping and precipitation depend on folding of hydrophobic domains, it has been proposed that their proteolytic hydrolysis might be an appropriate solution; however, this could lead to the production of highly hydrophobic peptides, no less difficult to recover than whole IMPs themselves (5).

The aim of this study was to set up a simple, effective, and reliable method (RM) to analyze MPs in a non-specialized proteomics laboratory setting. For benchmarking our work, we compared our method (RM) with a more conventional method for protein preparation in mass spectrometric analysis (CM) (6). To illustrate the favorable characteristics of RM, we characterized mouse band 3 (an IMP), mouse β-actin (an MA), the rat eight-transmembrane-strand MLC1 (megalencephalic leukoencephalopathy), and malaria parasite *Plasmodium berghei* pbSEP1, an IMP with one transmembrane portion. Finally, we used both RM and CM to identify rat lipid-raft membrane proteins and compared the respective effectiveness of the two methods.

Materials and Methods

Reagents and chemicals. SDS and ammonium bicarbonate (NH_4HCO_3) were obtained from INC Biochemicals (Milwaukee, WI, USA). Iodoacetamide (IAA) and cyanogen bromide (CNBr) were purchased from Fluka (Milwaukee, WI, USA), 1,4-dithiothreitol (DTT) and α-cyano-4-hydroxycinnamic acid matrix (CHCA) from

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Sigma Aldrich (St. Louis, MO, USA), trypsin was obtained from Promega (Madison, WI, USA), *N*-octyl β-D-glucopiranoside (OBG) from Pierce (Rockford, IL, USA), sodium chloride (NaCl) from Sigma Aldrich, lympholyte was from Cedarlane Laboratories Limited (Burlington, Ontario, Canada). Dulbecco's phosphate-buffered saline (D-PBS) from Invitrogen (Madison, WI, USA), and urea from Sigma Aldrich. Formic acid and isopropanol were purchased from Merck (Darmstadt, Germany), acetic acid from BDH (Milan, Italy) and methanol from Carlo Erba (Milan, Italy). All chemicals and solvents were analytical grade and were used as received. Trifluoroacetic acid (TFA) and water for chromatography were from J.T. Baker (Deventer, Netherlands), acetonitrile from Riedel-De-Haen (Seelze, Germany), chloroform from Sigma Aldrich.

Electrophoresis materials and instrumentation. Electrophoresis cells, polyacrylamide gels and reagent for gel electrophoresis (MOPS running buffer, loading buffer, reducing agent) were purchased from Invitrogen and ZipTip pipette tips for microvolume sample preparation of peptides prior to (MALDI-TOF) mass spectrometry (MS) were obtained from Millipore (MA, USA). All MS analyses were performed with a Voyager-DE STR MALDI-TOF instrument (Applied Biosystems, SCIEX, Toronto, Canada).

Purification and solubilization of erythrocyte membranes. Mouse blood (from CD1 mice) was depleted of leukocyte-rich plasma by centrifugation at 1500 ×g for 15 min at 4˚C. The resulting pellet was diluted in D-PBS containing complete protease inhibitor cocktail Roche (Mannheim, Germany). Following addition of lympholyte and centrifugation (800 ×g for 20 min), resting leukocytes were completely removed. The resulting packed red blood cells (RBCs) were washed repeatedly in 150 mM NaCl. In order to obtain RBC ghosts, cells were subjected to five consecutive freeze/thaw cycles in liquid nitrogen (7), followed by centrifugation at 22,000 ×g for 90 min at 4˚C. The resulting supernatant was discarded and RBC membranes were repeatedly resuspended in 5 ml of ice-cold phosphate buffer, pH 8, and centrifuged at 9,000 ×g for 20 min at 4˚C, until the supernatant appeared colorless. Centrifugation was finally increased to 20,000 ×g and washings were repeated until the ghost membranes appeared whitish-yellow. Membranes were stored at –80˚C. RBC membrane samples were dissolved in 100 μl of each of three types of buffer: i) 2% SDS 2% in 25 mM NH_4HCO_3 (8); ii) 2% OBG in 25 mM NH_4HCO_3 (3); iii) 6 M urea, 2 M thiourea (9).

Delipidation of membrane protein samples (RM). To remove the lipid components of lipoproteins in RBC membrane samples, the water/chloroform/methanol protein precipitation method was used (10). Briefly, 4 volumes of methanol, 1 volume of chloroform and 3 volumes of water were added to the dissolved RBC membranes and thoroughly mixed. Samples were then centrifuged at 14,000 ×g for 1 min. After water phase removal (the proteins being in the interphase), 4 volumes of methanol were added to the samples, which were thoroughly mixed and centrifuged at 14,000 ×g for 2 min. The supernatant was discarded and the protein precipitates were air dried for 1 h. To achieve better homogenization, the delipidized pellets were dissolved as described above at 70˚C for 2 h.

Reduction and alkylation of membrane protein samples (RM). DTT (1 μg) was added to 50 μg of RBC membrane proteins and samples were incubated for 30 min at room temperature (RT). Reduced proteins were alkylated by adding 5 μg IAA per 50 μg of proteins (20 min, RT).

SDS-PAGE electrophoresis (RM, CM). Samples to be separated by SDS-PAGE were treated as follows: 10 μl of loading buffer (containing β-mercaptoethanol, SDS, glycerol, TRIS-HCl and bromophenol blue) were added to 30 μl of RBC membrane proteins and the mixture was heated for 10 min at 70˚C. The samples were loaded onto a precast, gradient, 4-12% polyacrylamide gel using MOPS running buffer containing 4 μl of reducing agent (1×), as per manufacturer's instructions. The composition of the reducing agents is proprietary and undisclosed. The gel was fixed in a 40% methanol, 10% acetic acid solution and stained with colloidal Coomassie blue.

In-gel desalting (RM). Band 3 and β-actin protein bands were excised from the gel and transferred into 0.5 ml tubes. The gel spots were desalted through four step: i) 1 ml of deionized water was added and drained out after 5 min at RT; ii) the washing was repeated with the incubation time increased to 10 min; iii) 1 ml of 40% CH_3CN, 60% H_2O was added and drained out after 10 min at RT; iv) gel bands were dehydrated with 100 μl of 100% CH_3CN and desiccated in a vacuum concentrator.

In-gel trypsin/CNBr digestion (RM). The gel bands were destained in 100 μl of 50% ACN, 0.1% TFA and covered with 150 μl of acetonitrile until the gel pieces shrunk. Acetonitrile was removed and gel particles dried by centrifugation under vacuum. In-gel digestion was performed by adding 15 ng/μl trypsin in 25 mM ammonium hydrogen carbonate in quantities sufficient to moisten the gel sample spots (excess solution was eliminated). The well-hydrated samples were then incubated at 37˚C overnight under stirring, after which 25 μl of CNBr (5 mg/ml) in 70% TFA were then added to the gel slices and the samples were then stored in the dark at RT for 14 h.

After 14 h, the gel was separated from the hydrolysis solution and treated with 50 μl of 10% formic acid in isopropanol (v/v) for 10 min at RT. The solution was then collected and the operation was repeated by increasing the incubation time to 30 min. Thereafter, 20 μl of pure formic acid were added to the sample that was allowed to stand for 15 min at RT and the supernatant was collected and saved. Finally, the gel spots were washed with 20 μl of isopropanol and further incubated for 15 minutes at RT. All the washes containing peptides were combined and dried in a vacuum concentrator. The residues were resuspended in 5 μl of 50% acetonitrile, 0.1% TFA under Vortex agitation.

In-gel reduction/alkylation and trypsin digestion (CM). Band 3 and β-actin protein bands were excised from the gel, transferred into 0.5 ml tubes, destained in 50 mM ammonium hydrogen carbonate/acetonitrile 1:1 and covered with acetonitrile until the gel pieces shrank. Acetonitrile was removed and gel particles dried by centrifugation under vacuum. Proteins were reduced (10 mM DTT, 25 mM ammonium bicarbonate) for 30 min at 56˚C, cooled to RT and alkylated (55 mM IAA, 25 mM ammonium bicarbonate) for 30 min in the dark at RT. Gel pieces were washed in 50 mM ammonium hydrogen carbonate/acetonitrile 1:1 for 15 min and covered with acetonitrile until they shrank. Acetonitrile was removed and gel particles dried by centrifugation under vacuum. In-gel digestion was performed by adding 12.5 ng/μl trypsin in 25 mM ammonium carbonate at 37˚C overnight under continuous stirring. The supernatant was then harvested using a micropipet and stored at –20˚C.

MALDI/MS Analysis and data processing. Samples (1 μl) were applied to the target and air dried. Subsequently, 1 μl of CHCA (10 mg/ml) in 50% acetonitrile, 0.1% TFA (v/v) was applied to the

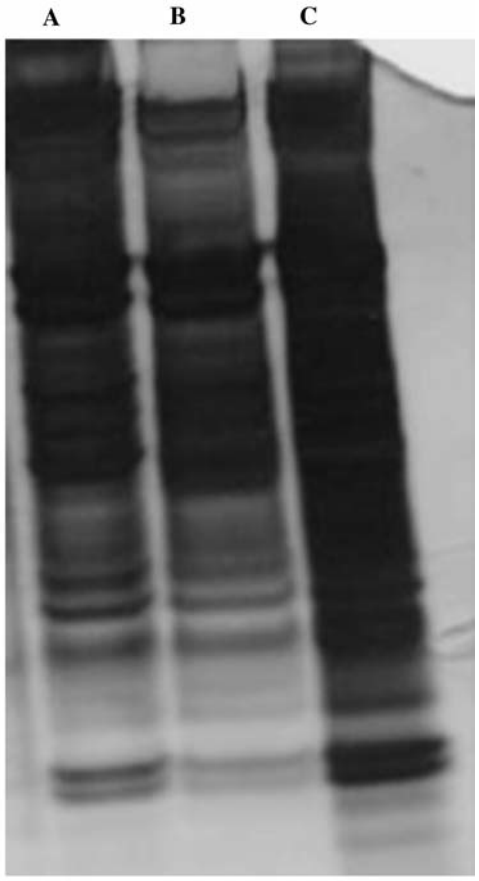


Figure 1. *1D SDS-PAGE of red blood cell membrane proteins solubilized with: A: 2% OBG in 25 mM NH_4HCO_3; B: 6 M urea, 2 M thiourea; C: 2% SDS in 25 mM NH_4HCO_3.*

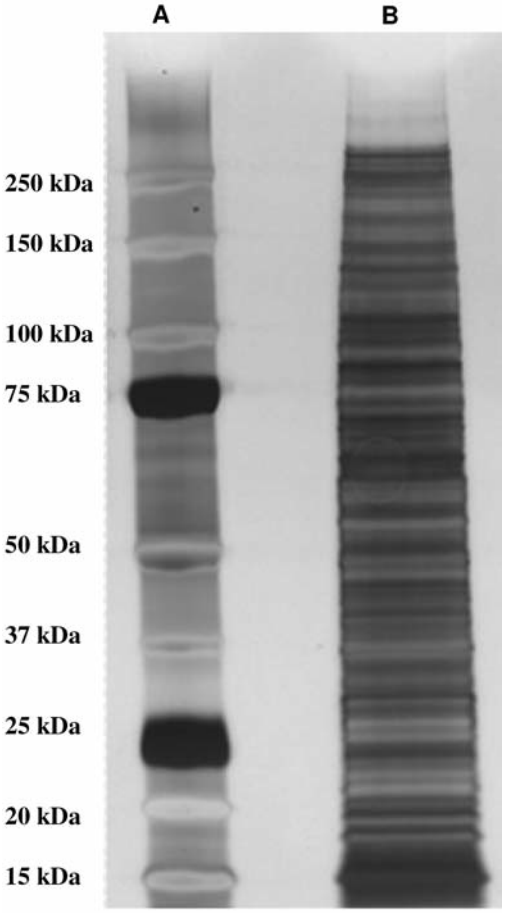


Figure 2. *1D SDS-PAGE of red blood cell membrane proteins purified by RM: A: protein markers; B: sample.*

sample and dried again. MALDI mass spectra were recorded using a Voyager-DE STR MALDI-TOF mass spectrometer using the reflectron mode. Ionization was performed with a 337 nm pulsed nitrogen laser. Spectra in the 750–4000 m/z mass range were accumulated and mass calibration was internally performed using molecular ions from matrix and trypsin autodigestion. Raw data were analyzed using Data Explorer software version 4.0.0.0 (Applied Biosystems) provided by the manufacturer and reported as monoisotopic masses.

The search engine Mascot peptide mass fingerprint (version 1.9.0; Matrix Science, London, UK) was used to process MS data, on a merged database for in-house search, which contained the predicted proteins from *Plasmodium berghei* genome (12,235 entries) downloaded from the ftp site of the Sanger Center (ftp://ftp.sanger.ac.uk), and the mouse (26,657 entries) and rat (21,179 entries) predicted proteins from the NCBI ftp site (ftp://ftp.ncbi.nih.gov/genomes). Non-redundancy of selected entries was carefully checked. Search parameters for MS spectra included: a maximum of two incomplete tryptic cleavages allowed; peptide mass tolerance 20 ppm; monoisotopic mass, 1+ peptide charge state as CHCA protonation, alkylation of cysteine by carbamidomethylation as a fixed modification, and oxidation of methionine as a variable modification.

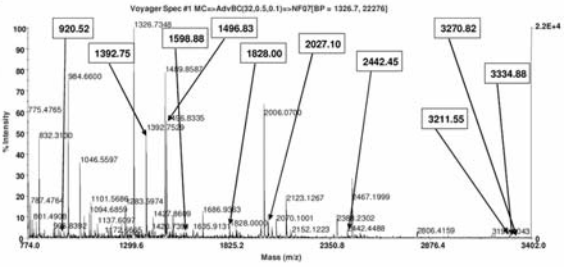
Results

With the aim of achieving optimal protein solubilization, we compared the proficiency of several buffers in extracting erythrocyte ghost proteins. Ghosts, *i.e.* empty cell membranes, are highly enriched in membrane proteins. The following three buffers were compared: A) 2% β-octyl glucopyranoside (OBG) in 25 mM NH_4HCO_3; B) 6 M urea/2 M thiourea in HEPES pH 8.0; C) 2% SDS in NH_4HCO_3 25 mM. As shown in Figure 1, the best recovery was obtained with buffer C, containing 2% SDS. Buffer B not only yielded a lower recovery but, in addition, produced poor mass spectra, due to difficult sample crystallization in CHCA (data not shown). It should be noticed that all of the tested buffers, including buffer C, produced a blurred migration pattern in 1D SDS-PAGE (Figure 1), presumably due to the presence of a significant lipid component. One drawback of the SDS-containing buffer was its tendency to allow protein refolding, due to the formation of an SDS gradient through the electrophoretic field,

A

RM-1 B3AT_MOUSE P04919
Total Sequence Coverage 33%
Sequence Coverage of the transmembrane portion 37%

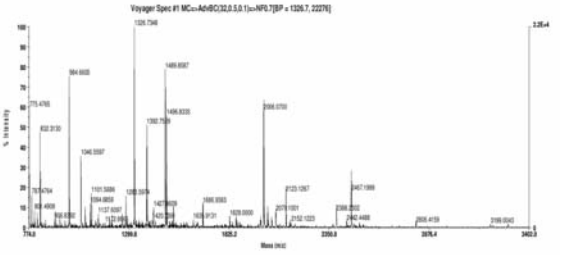
MGDMRDHEEV **LEIPDR**DSEE ELENIIGQIA YRDLTIPVTE MQDPEALPTE
QTATDYVPSS TSTPHPSSGQ VYVELQELMM DQR**NQELQWV EAAHWIGLEE**
NLREDGVWGR PHLSYLTFWS LLELQKVFSK **GTFLLGLAET SLAGVANHLL**
DCFIYEDQIR PQDREELLRA LLLKR**SHAED LGNLEGVKPA VLTRSGGASE**
PLLPHQPSLE TQLYCGQAEG GSEGPSTSGT LKIPPDSETT LVLVGRANFL
EKPVLGFVRL KEAVPLEDLV LPEPVGFLLV LLGPEAPHVD YTQLGR**AAAT**
LMTERVFR**IT ASMAHNR**EEL LRSLESFLDC SLVLPPTDAP SEK**ALLNLVP**
VQKELLRRRY LPSPAKPDPN LYNTLDLNGG K**GGPGDEDDP LRRTGRIFGG**
LIRDIRRRYP YYLSDITDAL SPQVLAAVIF IYFAALSPAV TFGGLLGEKT
RNLMGVSELL ISTAVQGILF ALLGAQPLLV LGFSGPLLVF EEAFFSFCES
NNLEYIVGR**A WIGFWLILLV MLVVAFEGSF LVQYISR**YTQ EIFSFLISLI
FIYETFSKLI KIFQDYPLQQ TYAPVVM**KPK PQGPVPNTAL FSLVLMAGTF**
LLAMTLRKFK **NSTYFPGK**LR RVIGDFGVPI SILIM**VLVDS FIKGTYTQKL**
SVPDGLKVSN SSARGWVIHP L**GLYRLFPTW** MMFASVLPAL LVFILIFLES
QITTLIVSKP ERKMIKGSGF HLDLLLVVGM GGVAALFGMP WLSATTVR**SV**
THANALTVMG KASGPGAAAQ IQEVKEQRIS GLLVSVLVGL SILMEPILSR
IPLAVLFGIF LYMGVTSLSG IQLFDRILLL FKPPKYHPDV PFVKRVKTWR
MHLFTGIQII CLAVLWVVKS TPASLALPFV LILTVPLRR**L ILPLIFRELE**
LQCLDGDDAK VTFDEENGLD EYDEVPMPV



B

CM-1 B3AT_MOUSE P04919
Total Sequence Coverage 30%
Sequence Coverage of the transmembrane portion 0%

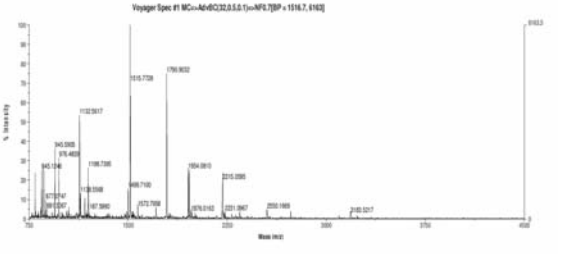
MGDMRDHEEV **LEIPDR**DSEE ELENIIGQIA YRDLTIPVTE MQDPEALPTE
QTATDYVPSS TSTPHPSSGQ VYVELQELMM DQR**NQELQWV EAAHWIGLEE**
NLREDGVWGR PHLSYLTFWS LLELQKVFSK **GTFLLGLAET SLAGVANHLL**
DCFIYEDQIR PQDREELLRA LLLKR**SHAED LGNLEGVKPA VLTRSGGASE**
PLLPHQPSLE TQLYCGQAEG GSEGPSTSGT LKIPPDSETT LVLVGRANFL
EKPVLGFVRL KEAVPLEDLV LPEPVGFLLV LLGPEAPHVD YTQLGR**AAAT**
LMTERVFR**IT ASMAHNR**EEL LRSLESFLDC SLVLPPTDAP SEK**ALLNLVP**
VQKELLRRRY LPSPAKPDPN LYNTLDLNGG K**GGPGDEDDP LRRTGRIFGG**
LIRDIRRRYP YYLSDITDAL SPQVLAAVIF IYFAALSPAV TFGGLLGEKT
RNLMGVSELL ISTAVQGILF ALLGAQPLLV LGFSGPLLVF EEAFFSFCES
NNLEYIVGR**A** WIGFWLILLV MLVVAFEGSF LVQYISRYTQ EIFSFLISLI
FIYETFSKLI KIFQDYPLQQ TYAPVVMKPK PQGPVPNTAL FSLVLMAGTF
LLAMTLRKFK **NSTYFPGK**LR RVIGDFGVPI SILIMVLVDS FIKGTYTQK**L**
SVPDGLKVSN SSARGWVIHP LGLYRLFPTW MMFASVLPAL LVFILIFLES
QITTLIVSKP ERKMIKGSGF HLDLLLVVGM GGVAALFGMP WLSATTVR**SV**
THANALTVMG KASGPGAAAQ IQEVKEQRIS GLLVSVLVGL SILMEPILSR
IPLAVLFGIF LYMGVTSLSG IQLFDR**ILLL FKPPKYHPDV PFVK**RVKTWR
MHLFTGIQII CLAVLWVVKS TPASLALPFV LILTVPLRR**L ILPLIFRELE**
LQCLDGDDAK VTFDEENGLD EYDEVPMPV



C

RM-2 ACTIN BETA MOUSE Q3U5R4
Total Sequence Coverage 44%

MDDDIAALVV DNGSGMCK**AG FAGDDAPRAV FPSIVGRPRH QGVMVGMGQK**
DSYVGDEAQS KRGILTLKYP IEHGIVTNWD DMEK**IWHHTF YNELRVAPEE**
HPVLLTEAPL NPKANREKMT QIMFETFNTP AMYVAIQAVL SLYASGRTTG
IVMDSGDGVT HTVPIYEGYA LPHAILRLDL AGR**DLTDYLM K**ILTER**GYSF**
TTTAEREIAR DIKEK**LCYVA LDFEQEMATA ASSSSLEKSY ELPDGQVITI**
GNERFRCPEA LFQPSFLGME SCGIHETTFN SIMKCDVDIR **KDLYANTVLS**
GGTTMYPGIA DRMQKEITAL APSTMKIK**II APPERK**YSVW IGGSILASLS
TFQQMWISKQ EYDESGPSIV HRKCF



D

CM-2 ACTIN BETA MOUSE Q3U5R4
Total Sequence Coverage 45%

MDDDIAALVV DNGSGMCK**AG FAGDDAPRAV FPSIVGRPRH QGVMVGMGQK**
DSYVGDEAQS KRGILTLKYP IEHGIVTNWD DMEK**IWHHTF YNELRVAPEE**
HPVLLTEAPL NPKANREKMT QIMFETFNTP AMYVAIQAVL SLYASGR**TTG**
IVMDSGDGVT HTVPIYEGYA LPHAILRLDL AGRDLTDYLM KILTER**GYSF**
TTTAEREIAR DIKEK**LCYVA LDFEQEMATA ASSSSLEKSY ELPDGQVITI**
GNERFRCPEA LFQPSFLGME SCGIHETTFN SIMKCDVDIR **KDLYANTVLS**
GGTTMYPGIA DRMQKEITAL APSTMKIK**II APPER**KYSVW IGGSILASLS
TFQQMWISKQ EYDESGPSIV HRKCF

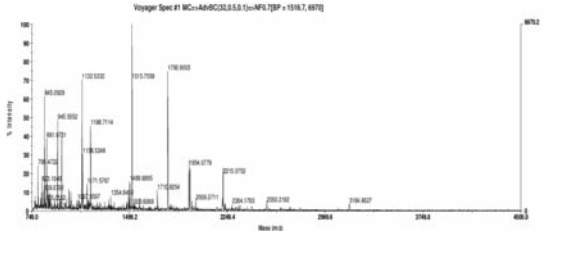


Figure 3. *Comparison between the sequence coverage of mouse band 3 and β-actin analyzed by RM (RM-1 and RM-2, respectively; A and C) and CM (CM-1 and CM-2, respectively; B and D). Transmembrane portions are underlined; detected peptides are highlighted in bold (A). The corresponding MALDI/MS mass spectra from each panel are shown next to the sequences. Arrows in A: peaks corresponding to transmembrane portions of band 3.*

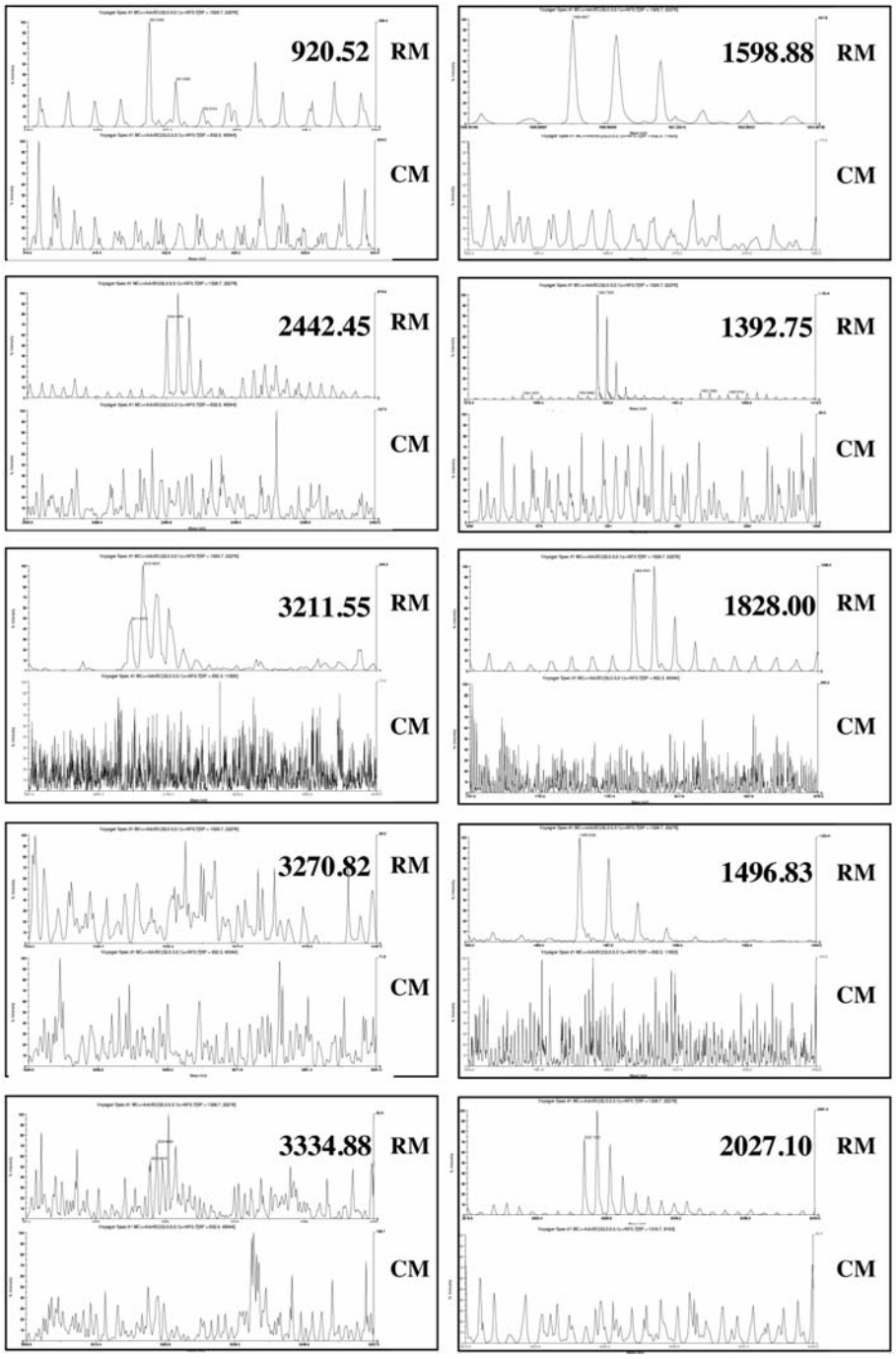
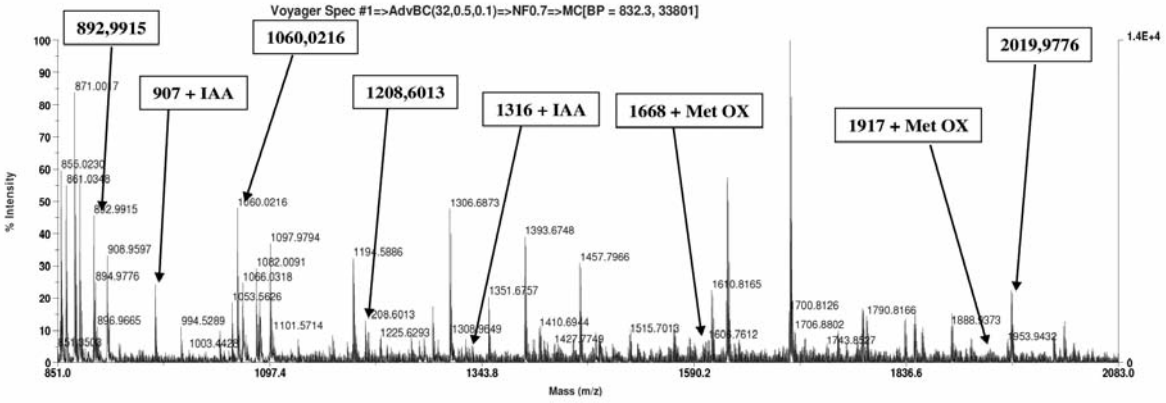


Figure 4. *Enlarged mass range region containing each of the hydrophobic peptide peaks acquired from proteins prepared by RM and the corresponding mass range from CM spectra*.

causing incoherent protein migration and band blurring. In order to avoid interferences caused by lipids, a chloroform/methanol lipid-extraction/protein-precipitation step was performed and the proteins successively recovered were fractionated on a 1D SDS gel. To prevent partial in-gel protein refolding due to intra- and intermolecular disulfide bond formation, the SH groups were reduced with DTT and alkylated with IAA prior to the electrophoretic run. This resulted in a sharpened, well-defined 1D migration pattern, as shown in Figure 2. Furthermore, complete protein denaturation facilitated the digestion of the hydrophobic cores, as they were not shielded from the enzyme by the hydrophilic portions.

MLC1 RAT
Total Sequence Coverage 20%

MTREGQFREE LGYDRMPTLE RGRQDAGRQD TGSYTPDTKP KDLQLTKRLP PCFSYKTWVF
SVLMGSCLLV TSGFSLYLGN VFPSEMDYLR CAAGSCIPSA IVSFAVGRRN VSAIPNFQIL
FVSTFAVTTT CLIWFGCKLI LNPSAININF NLILLLLLEL LMAATVIISA RSSEEPCKKK
KGSISDGTNI LDEVTFPARV LKSYSVVEVI AGVSAVLGGV IALNVEEAVS GPHLSVTFFW
ILVACFPSAI ASHVTAECPS KCLVEVLITI SSLTSPLLFT ASGYLSFSVM RIVEIFKDYP
PDIKSYDVLL LLLLLLLLLQ GGLNTGTAIQ CVSFKVSARL QAASWDSQSC PQERPAGEVV
RSPLKEFDKE KAWRAVVVQM AQ



pbSEP1 *P berghei*
Total Sequence Coverage 40%

MKLAKALYFV AFLLAIKVLT PGSNNYVEAK PANSKKVTKS GDNAFIKKIK
NNKAAFISTL AATVALAIAT TFGVMHYQNN GNDKKKPSGL DGKTPLIIPR
KKTPSASDND SVPPS

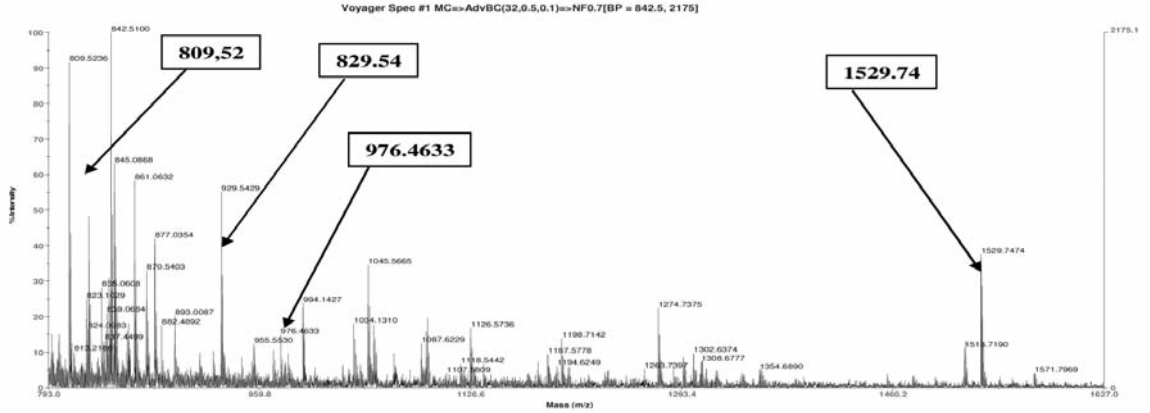


Figure 5. *Amino acid protein sequence and MALDI mass spectra acquired from MLC1 and pbSEP1 proteins prepared with RM. Arrows indicate the peptides detected.*

In order to maximize recovery of both hydrophobic and hydrophylic peptides, we subjected the gel slice to an extraction procedure. The CNBr supernatant was removed and stored and the gel slice was sequentially extracted with different solvents (see Materials and Methods). The CNBr supernatant previously set aside and the extraction buffers were pooled and dried using a vacuum concentrator. Finally, the precipitated peptides were resuspended in 50% acetonitrile, 0.1% TFA for MALDI mass spectrometry analysis. This procedure allowed us to recover a significant number of hydrophobic peptides, with no reduction in overall sequence coverage. By way of example, Figure 3 shows the results obtained on the integral mouse band 3 protein (at least 1 transmembrane portion), with both the conventional preparation method (CM) and the procedure described here (RM). In the same Figure, β-actin is also shown in order to illustrate that the new procedure does not appreciably reduce sequence coverage of hydrophylic proteins. Furthermore, Figure 4, showing enlarged areas of each of the hydrophobic peptide peaks, shows that the peaks newly detected with our method are truly absent from the spectra obtained from protein prepared by the more common procedure.

To further test the method, we analyzed MLC1, a very hydrophobic rat protein with eight transmembrane domains isolated from rat astrocytes (11) and pbSEP1, a malaria parasite IMP with one transmembrane portion, which was isolated from the lipid rafts of infested erythrocytes (12). As shown in Figure 5, our method allowed identification of the two proteins with good confidence and specifically detected a number of highly hydrophobic peptides (highlighted in the Figure). On the contrary, the two proteins could not be identified when conventionally prepared (data not shown).

Since the method described here is particularly suited for hydrophobic protein detection, we applied it to the systematic identification of proteins located in lipid rafts, which are very hydrophobic membrane microdomains. As reported in Table I, our method compared favorably with a more widely used preparation as to both the number of identified proteins (up by 47%) and the sequence coverage, which was generally slightly increased.

Table I. *Comparison between lipid-raft proteins identified using RM and CM.*

Proposed annotation IMP	ID	% Protein sequence coverage	
		With RM	With CM
Ankiryn	gi |191940|	32	26
Ras-related protein Rab-15	gi |27734454|	11	--
Transferrin receptor protein I	gi |27708038|	41	19
Solute carrier family 4	gi |6755560|	45	27
Similar to fatty acid transport protein	gi |20140576|	32	--
Solute carrier family 10	gi |24308414|	16	--
Galectin-5	gi |1346430|	66	60
Aquaporin-1	gi |6680710|	18	--
Alpha enolase	gi |38570129|	10	--
Heat-shock protein 8	gi |31981690|	8	4
Beta-spectrin	gi |2506246|	35	34
Peroxiredoxin2	gi |2499469|	14	--
Alpha-spectrin	gi |17380523|	28	27
Actin cytoplasmic (beta actin)	gi |46397334|	49	50
Hypotethical protein XP 485465	gi |13637776|	48	45
Erytrocyte membrane protein band 4.2	gi |1345604|	41	28
Biliverdin reductase B	gi |21450325|	16	--
Erythrocyte membrane protein band 4.1	gi |51709565|	36	25
Carbonic anhydrase II	gi |31981657|	59	59
Carbonic anhydrase I	gi |6753266|	47	47
Hemoglobin beta chain	gi|204352|	73	73
Hemoglobin alpha chain	gi| 1304381|	57	56

ID according to NCBI database.

Discussion

The method described here and summarized in Table II includes several steps that have been previously described or are parts of different procedures. However, we developed a method that optimally exploits each of these steps in order to maximize the successful recovery and identification of membrane proteins.

MPs are known to be poor in lysine and arginine residues, compared to hydrophilic proteins (13). Thus, the conventional trypsin digestion yields exceedingly long peptides, on average. This problem is often overcome by performing double trypsin/CNBr digestion (13, 14). The polypeptides generated by such digestion are, on average, neither too long nor too hydrophobic, so as to prevent undesirable peptide folding and ensuing precipitation in the hydrolysis buffer. However, the two digestions are performed in widely diverging conditions, trypsin requiring a near-neutral pH, while CNBr is active in very acidic solutions. To overcome the relative incompatibility of the two requirements, trypsin digestion was performed first in a volume of buffer just sufficient to rehydrate the gel slice (see Materials and Methods), to prevent excess buffer from modifying the pH of the CNBr solution applied later. An additional advantage of this procedure is to prevent hydrophobic peptides from diffusing out of the gel slice and folding and precipitating in the trypsin buffer. After trypsin digestion, CNBr hydrolysis is performed as usual.

We have shown that the procedure presented here is suitable for both identifying purified hydrophobic proteins and systematically investigating hydrophobic protein mixtures. It can be easily applied even in non-dedicated

Table II. *Comparison between RM and CM.*

	RM	CM
Delipidation of membrane protein sample	Chloroform/methanol	
Reduction and alkylation of membrane protein samples	Reduction: 1 μg DTT per 50 μg membrane protein, Alkilation: 5μg IAA per 50 μg protein (20 minutes, RT).	
SDS–PAGE electrophoresis	Sample: 10 μl loading buffer + 30 μl RBC membranes protein sample (10 minutes, 70˚C). Gel: Precast 4-12% polyacrylamide gel + MOPS running buffer + 4 μl reducing agent (1x) Fixing solution: methanol 40% + acetic acid 10%. Staining solution: colloidal coomassie blue.	
In-gel desalting	Deionized water (5 min, RT), acetonitrile 40% (10 min, RT). Dehydration: 100% acetonitrile. Dry in Speedvac	
In-gel trypsin/CNBr digestion	Trypsin: 15 ng/μl (16 h, 37˚C) CNBr (5 mg/ml) in 70% TFA (14 h, RT)	
In-gel reducing/alkylation and trypsin digestion		Destain: 50 mM ammonium bicarbonate /acetonitrile (1/1). Reduction: 10 mM DTT, 25 mM ammonium bicarbonate (30 min at 56˚C). Alkylation: 55 mM IAA, 25 mM ammonium bicarbonate (30 min dark, RT). In-gel digestion: 12.5 ng/μl trypsin in 25 mM ammonium bicarbonate (overnight 37˚C)
Peptide extraction from gel	Formic acid 10% in isopropanol (10 min, RT), formic acid (15 min, RT), isopropanol (15 minutes, RT), dry in Speedvac, solubilization in acetonitrile 50% /TFA solution 0.1%.	
Matrix-assisted laser desorption ionization mass spectrometry (MALDI/MS) analysis	Matrix: α cyano-4 hydroxycinnamic acid. MALDI: Peak list of monoisotopic (MH+) masses, obtained by peak deisotoping, mass range of 750-4000 m/z.	

RT, Room temperature; IAA, iodacetamide.

laboratories, such as those mostly devoted to clinical chemistry. It can be used to prepare proteins for mass-fingerprint identification, probably the easiest and most widely applied protein MS approach in non-specialized laboratories. While it does not impair hydrophylic protein identification and coverage, its strength lies in the analysis of IMPs, a set of proteins well known to cause a variety of analytical problems using a range of different techniques.

We believe this method to be an efficient and useful addition to the methodological arsenal available to modern protein mass spectrometrists.

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