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Evaluation of Macrophage Cell Membrane Preparation for Proteomics Analysis

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INTRODUCTION

In the proteomics science powerful technologies are applied for the comprehensive identification and characterization of membrane proteins. However the peculiarities of these molecules impose technical drawbacks. While 2-DE has a high capacity for separation and resolution allowing, for instance, visualization of isoforms and post-translation modified proteins, the highly hydrophobic, low abundant and basic proteins are not amenable to this approach. Non-gel methods have extended the analytical range capacity to include basic, hydrophobic and high molecular weight proteins. Yet, detection and profiling of peptides from transmembrane span regions is difficult, decreasing the confidence level of detection of embedded proteins. Regardless the tactic chosen, enrichments in concomitance with efficient extraction and solubilization are crucial.

In this study, we used J774 macrophages membrane samples to determine optimal extraction and solubilization conditions for the application of gel and non-gel methods.

MATERIALS & METHODS

Cell culture
J774 macrophages, a reticuloma cell line of murine origin, were grown at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum in 5 % CO_2. Cell were seeded in culture dishes (13 cm^2) at a density of ca. 5 x 10^6 cells per cm^2 and harvested after 2 days.

Preparation of membranes samples. J774 cell monolayers were rinsed with ice-cold PBS and after scraped with a policeman and collected (eight polled monolayers) in same buffer. Cells were centrifuged at 2,500 rpm for 10 min at 4 °C and cell pellets resuspended in 10 mM Tris HCl, pH 7.4 plus protease-inhibitor cocktail-EDTA free (one tablet per 10 mls). Cell were lysed on ice using a Dounce B homogenizer (glass/glass, tight pestle) applying 22 strokes. For crude membrane preparations, the homogenates were layered on top of 50 % (w/v, 1.5 M) sucrose solution plus 1 mM PMSF and centrifuged in a Beckman SW40 rotor at 280.000 x g for 1 h at 4 °C. The crude membrane fraction isolated from the interphase buoyant-sucrose was diluted to 8 ml with cold 100 mM Na_2CO_3, pH 11.0 (stripping) and after 15 minutes on ice loaded on top of a fresh 50% sucrose cushion and ultracentrifuged in the same conditions as described above. Alkaline-stripped membranes were recovered from the interphase and washed in 10 mM Tris-HCl, pH 7.4 plus protease-inhibitor-cocktail and. At this step an aliquot of sample was removed for protein quantification using the BCA kit (Pierce) and after centrifuged in a Type 50 Ti rotor at 100,000 x g for 20 min at 4 °C. Stripping is done for effective removal of peripheral associate membrane proteins and conversion of sealed vesicles into flat membranes releasing cisternal contents. Homogenates obtained from a monolayer rendered approximately 1 mg of proteins and 8 monolayers were necessary to collect approximately 2 mg of proteins from membranes preparations. Sucrose solutions for discontinuous gradients were prepared in 10 mM Tris-HCl, pH 7.4 plus 0.5 M PMSF. The sucrose step gradients were done by layering cold solutions of sucrose in a tube (14 x 95 mm, Ultra-clear™, Beckman) in the following order: 1.5 ml of 1.10 density solution, 1 ml of 1.13 density solution, 1 ml of 1.15 density solution, 2 ml of 1.17 density solution and 2 ml of 1.19 density solution with homogenates at on top. Gradients were ultracentrifuged in a Beckman SW40 rotor at 280.000 x g for 2 h at 4 °C. After this procedure the resulted visible bands at each of five interphases which were collected on ice (16 x 76 mm polycarbonate tube) and diluted to 6 ml with cold 0.1 M Na_2CO_3, pH 11 and centrifuged in a Type 50 Ti rotor at 100.000 x g for 30 min at 4 °C. The pellets were resuspended in 10 mM Tris-HCl, pH 7.4 plus protease-inhibitor cocktail-EDTA free and aliquots were removed for protein content determination.

SDS-PAGE and Western Blotting. Membrane protein samples were loaded on 7.5 % acrylamide gels, without boiling, in Laemmli buffer. Proteins were transferred onto a nitrocellulose membranes that then were blocked with 3 % milk in TBST (20 mM Tris-HCl, 500mM NaCl and 0.05% Tween 20 at pH 7.5) over night at 4 °C. Subsequently, blots were incubated with primary antibodies, the anti-MRP1/Abcc1 at 1:2000, anti-prohibitin at 1:1000 and anti-actin 1: 1500 dilution respectively. The secondary antibodies were horseradish peroxidase (HRP) conjugated and bands were visualized by the enhanced chemiluminescent method.

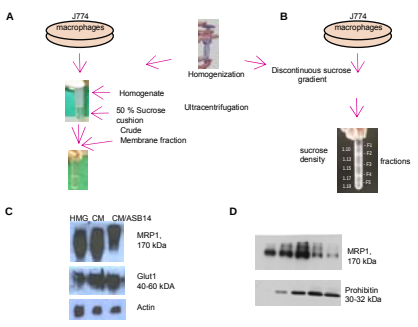
Isoelectric focusing (IEF) and second-dimensional gel. Strips were actively rehydrated in presence of sample. Equilibration was done for 20 min in solutions containing 2 % DTT and 2.5 % iodoacetamine in two consecutively steps. Proteins in the second dimension gels were visualized using Sypro Ruby stain (Sigma).

Image acquisition and analysis
Imaging of Sypro Ruby stained 2DE gels were done in a Bio-Rad Gel Doc 2000 documentation system equipped with a CCD camera with a pixel density (gray levels) of 8 bit. The images were analyzed with the PDQuest Software from Bio-Rad. Western Blot X-ray films were scanned using a GS800 scanner from Bio-Rad.

Tryptic digestions and MALDI-TOF. Protein spots were manually excised and in-gel digested overnight at 37°C with 0.02 µg of modified trypsin (Promega) in 50 mM ammonium bicarbonate and peptides were extracted with 60% ACN/ 0.1% formic acid in water, dried and dissolved in 10µl of 0.1 % formic acid. The MALDI plate was manually spotted and tryptic peptides were analyzed by MALDI-MS using a Micromass A@ldi™ spectrometer (Manchester, UK). Previous to in-solution tryptic digestion with 0.1 µg/µl trypsin, reduction was done with 10 mM DTT (in 5 µg/µl sample) for 30 min at 60°C and subsequently alkylation with 100 mM iodoacetamide for 30 min.

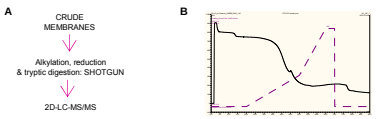
2D-LC separations. The 2D-LC were performed on an Ultimate Dual-Gradient Capillary/Nano LC system (Dionex-LC Packings). In the first dimension, 40 µg of digested proteins were separated using a 0.3 mm x 150 mm column packed with POROS 10S cation exchange resin. The capillary LC pump operated at a flow rate of 5 µL/min and generated a linear gradient, starting 20 min after injection, of 0 – 40% B over 30 min, followed by a steeper gradient of 40 – 100% B in 15 min. Buffer A was 5mM KH_2PO_4, 5% acetonitrile (ACN), pH 3 in water, whereas buffer B was the same buffer with 500 mM KCl. In the second dimension, separations were performed on a RP-nano-LC-column (Zorbax 300SB-C18, 3.5µm, 150mm x 75µm. Agilent) using a linear elution gradient from 5 to 80% ACN in 25 min at a flow rate of 250nl/min. Fractionation was performed by spotting the effluent directly on a MALDI target plate at 30s intervals using the same Probot device. MALDI target plates were prespotted with 0.4 µl matrix solution (10mg/ml alfa-cyano-4-hydroxycinnamic acid in 50% ACN, 0.1% TFA (v/v) in water containing 10mM dibasic ammonium citrate), and after fractionation, 0.1 µl of a saturated matrix solution (in 50% ACN, 20%

Scheme of protocols for preparation of crude membranes and plasma membrane enriched proteins



Crude membranes were obtained by ultracentrifugation of cell homogenates layered on top of 50% sucrose cushions and isolating the sample from interphase (**A**). The crude membranes were evaluated by Western blot for the presence of intrinsic membrane proteins, MRP1 and Glut1 and the soluble protein actin (**C**). To decrease the levels of contaminating soluble proteins and to enrich in plasma membrane proteins, homogenates were separated on discontinue sucrose gradients. (**B**) From each interphase, sample fractions were analyzed by Western blot for the expression of the plasma membrane protein MRP1 and prohibitin, a mitochondria protein marker which localized in the inner membrane (**D**). HMG, homogenate resuspended in 10 mM Tris HCl, pH 8.0. CM, crude membranes. CM/ASB14, proteins collected by ultracentrifugation after extraction with 10% ASB14. The detergent was after clean up using with the Amersham 2D clean up kit 7 M urea, 2 M thiourea, 1.2% CHAPS and 0.4% ASB14.

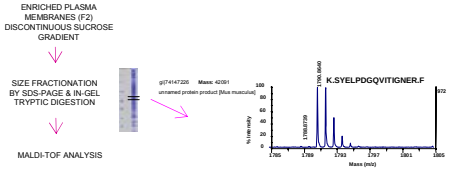
Bottom-up Shotgun 2D-LC MS/MS of crude macrophage membrane preparations



PROTEIN NAME	ACCESSION NUMBER	LOCATION	PEPTIDE
BIT (Tyrosine phosphatase P84 Precursor), CD172a antigen	P97797	Plasma membrane, single pass	77-89 LLIYSFTGEHFPR
			488-509 AQPAPKPEPSFSEYASV
CD44 antigen	P15379	Plasma membrane, single pass	32-40 YAGVFHVEK
Voltage-dependent anion channel 2 VDAC2	P45880	Outer mitochondrial membrane protein porin 2	279-295 SFNAGGHKLGLALELEA
Fc γ b1, CD32	P08101	Plasma membrane, single pass	160-175 TLHQSKPVTITVQGPK
			120-127 ISFFHNEK
Prohibitin, B-cell receptor-associated protein 32, BAP32	P67778	Mitochondrial inner membrane	158-177 AATFGLILDDVSLTHLTFG
Sideroflexin 1	Q99JR1	Mitochondrial membrane, multipass	25-35 ASHFFTVTDPR

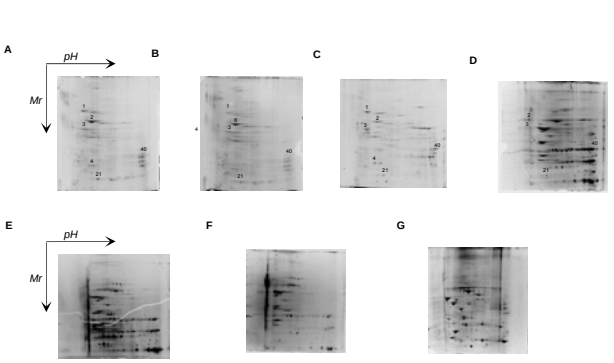
Crude J774 macrophages membrane samples treated with a solubilization cocktail which included 2% C7BzO were reduced (10 mM DTT), alkylated (100 Mm iodacetamide) and digested in-solution with trypsin previous injection to a 2D-LC (**A**). The 2D-LC system consist of a capillary LC pump for the separation in the first dimension and a nano LC pump for the separation in the second dimension. Peptides eluted from first column are fractionated in aliquots at 2 min interval in micro-title plates. An autosampler loads the aliquots onto a RP trapping column. The samples are then eluted onto the analytical separation column and the eluent is spotted directly on a MALDI target using a Prorobot device. Chromatogram showing UV traces from the SCX runs of the crude membrane samples (**B**). The table shows relevant proteins identified after MALDI/MS analysis of the fraction 15 of the SCX including the three underlined plasma membrane proteins.

1D-MALDI/MS approach for enriched plasma membrane sample fractions



Workflow of the 1D-MS approach. The membrane fraction F2 was isolated from the interphase between sucrose densities of 1.10 and 1.13 of a discontinuous gradient (**A**). Mass spectrum of a peptide (sequence at top right corner) resulted from the in-gel tryptic digestion of a arbitrarily selected SDS-PAGE band (**B**).

Extraction and solubilization optimization of crude membrane samples from J774 macrophages separated by 2DE



SPOT NUMBER	PROTEIN NAME	ACCESION NUMBER	LOCALIZATION
1	78 kDa glucose regulated protein GRP78	P06761	Endoplasmic lumen
2	Heat shock protein chaperonin	P63038	Mitochondria matrix
3	ATP B synthase β chain	P10179	Mitochondria
19	Prohibitin B receptor α chain associated protein 32	P03265	Mitochondria inner membrane
21	ATP B synthase α chain (fragment)		Mitochondria
40	Malate dehydrogenase	P08249	Mitochondria matrix

EXTRACTION AND SOLUBILIZATION COKTAILS

I. 10 mM Tris HCl, pH 8; 7 M urea, 2 M thiourea, 5 mM TBP (tributylphosphine), and 5 % ASB14 (amidosulfobetaine-14)
II. 10 mM Tris HCl, pH 8; 7 M urea, 2 M thiourea, 5 mM TBP and 10 % ASB14
III. 10 mM Tris HCl, pH 8; 7 M urea, 2 M thiourea, 5 mM TBP and 15 mM DHPC (1, 2-diheptanoyl-*sn*-glycero-3-phosphatidyl choline)
IV. 10 mM Tris HCl, pH 8; 7 M urea, 2 M thiourea, 5 mM TBP and 2% C7BzO 3-(4-heptyl)phenyl-3-hydroxypropyl dimethylammonio propanesulfonate)
V. 10 mM Tris HCl, pH 8; 7 M urea, 2 M thiourea, 5 mM TBP, 2% C7BzO and 5% ASB14
VI. 10 mM Tris HCl, pH 8; 7 M urea, 2 M thiourea, 5 mM TBP, 15 mM DHPC, 2% C7BzO and 2% ASB14
VII. 10 mM Tris HCl, pH 8; 7 M urea, 2 M thiourea, 5 mM TBP 2% C7BzO, 2% ASB14, 15 mM DHPC and 2% DDM dodecyl- β-D-maltose)

Differences in the 2DE patterns after extraction and solubilization of membrane proteins using various cocktails. The crude membrane samples (300 µg) were focused on 17 cm IPG strips, pH 3-10 NL (Bio-Rad) in a Protean IEF System (BioRad) at 250 V for 30 min, 4000 V for 6 hr and 8000 V for 3 hr and 8000 V up to 50 kVh reaching, approximately, 90000kVh at end. Equilibration of strips was performed using the DTT-iodoacetamide two-step method. In the second dimension IPG´s were applied on 7.5 % acrylamide gel and stained with Sypro Ruby protein gel stain. Imaging was done with the Gel Doc™ documentation system (BioRad). The macrophage crude membrane samples were treated with the respective cocktail (Table, bottom right) a follow: cocktail **I** in gel **A**, **II** in **B, III** in **C, IV** in **D, V** in **E, VI** in **F** and **VII** in **G**. Numbered spots correspond to major identified proteins and described in the Table (top right). Protein spots were manually excised and tryptic digested analyzed by MALDI-MS using a Micromass A@ldi™ spectrometer (Manchester, UK).

RESULTS & DISCUSSION

Two membrane samples were used: crude and plasma membrane enriched. For the optimization of extraction and solubilization, we tested various cocktails on crude membrane preparations. Also, a non-gel based approach was assessed in crude membrane preparations. The plasma-membrane enriched samples were separated by SDS-PAGE or 1D (one dimension) and tryptic peptides identified by MALDI/MS.

Various cocktail were evaluated for the ability to render high number of clear and well focused protein stops on 2DE gels. The widely used detergent ASB14 did not offer a satisfactory extraction power at any of the concentrations tested (5 and 10 %). DHPC was a more effective reagent based on the number of spots and the sharp protein pattern displayed (around 150 protein spots). However, DHPC induces a two-phase separation (detergent-aqueous) at room temperature. In this case, it would be required to collect the detergent phase, to precipitate and solubilize the proteins once more. The addition of these steps could jeopardize the experimental reproducibility, thus the DHPC was excluded. With the detergent C7BzO it was attained the best gel quality and it did not partitioned at room temperature. The C7BzO performance could not be improved with the addition of ASB14 or DDM.

J774 macrophage crude membranes were treated with C7BzO and analyzed by a shotgun-2D-LC MS/MS approach. We did not encounter detergent compatibility problems when proteins were in-solution digested with tryptsin neither during the 2D-LC separation. The fraction 15 of the SCX was further analyzed by MALDI/MS and among the identified peptides (from 15 different proteins) there were three corresponded to plasma membrane proteins.

Enriched plasma membrane samples were study by 1D-MALDI//MS. The in-gel tryptic digestion and mass spectrometry analysis proceeded without interference from the detergent present in the sample.

CONCLUSIONS

The proteomics field has the potential to bring up important contributions to understand vital biological functions of the membrane proteins. To enable such goal, it might be required the simultaneous application of complementary analytical strategies to tackle the peculiar chemical characteristics of these proteins. Here, we tested and assessed a relevant membrane model, the macrophage membrane. The protein samples extracted and solubilized in various conditions were evaluated by the 2DE gel profiles (spot number, focusing, resolution). As compared to ASB14, DHPC and DDM, C7BzO allowed superior solubilization power and probed to be suitable for MS-based analysis. The samples prepared in C7BzO- based cocktail were compatible with in-gel and in-solution tryptic digestions. Furthermore, crude membrane and enriched plasma membrane samples treated in similar fashion were analyzed by 2D-LC-MS/MS and 1D-MS, respectively. We had successfully extracted and identified membrane proteins from organelles and plasma membrane. These series of experiments represent a probe of concept to extent these studies to other approaches, for instance quantitative strategies such as, the Stable Isotope Labeling with Amino acids in Cell culture (SILAC).