Development of a Protocol for extraction and Preparation of Macrophage Integral Membrane Proteins for Proteomics Analysis by the Conventional 2DE-MS Approach

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INTRODUCTION

Membrane proteins are targets of prime interest to the pharmaceutical field largely because of their major role as receptors or drug transporters. Furthermore, the importance of membrane transporters in the pharmacokinetics and tissue distribution (bioavilability) of many therapeutics become recently evident, as well as, the remarkable action of efflux pumps in the development of drug resistance.

The application of the classical high-resolution 2-DE (two dimensional gel electrophoresis) separation technique and mass spectrometry methods for identification of proteins showed to be a very successful tactic to study proteins expressed in a system. While the characterization of abundant and soluble proteins through such proteomics approaches continue revealing new proteomes, membrane proteins remain extremely difficult to analyze in this way. In particular proteins embedded in the lipid bilayer, like transporters possessing variable number of transmembrane spans (TMS) are underrepresented or no present in the 2DE profiles. The difficulties that hamper or even prevent their characterization reside in their very intrinsic properties: extreme hydrophobicity and poor solubility.

Here, we standardized a protocol for extraction of proteins from membrane preparations from the murine macrophages cell line J774 and their analysis by 2-DE and MS. And, we evaluate the efficiency of the nonionic detergent ASB14 to solubilize proteins with 17 (MRP1, multidrug transporter) and 12 TMS (Glut1, glucose transporter) by Western Blot.

MATERIALS and METHODS

Cell line. J774 macrophages, a mouse cell line, was maintained at 37°C, in a 5 % CO_2 atmosphere, in RPMI medium supplemented by 10 % fetal calf serum. Cultures were initiated at a density of ca. 2 x 10^5 cell per cm (140 cm^2 dishes) and used after 2 days when they reached confluence.
Preparation of membrane fraction. Cell monolayers were washed with ice-cold PBS and after scraped with a policeman. Cells were centrifuged at 2,500 rpm for 10 min at 4 °C washed in PBS and collected in TS buffer (10 mM Tris Cl, pH 7.4, 250 mM sucrose). Cell lysates were prepared using a Douce B homogenaizer (glass/glass, 20 strockes) in presence of protease inhibitors (1 mM PMSF, 1 mM sodium orthovanadate, 1 µg/ ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A) layered on top of a 50 % sucrose solution and ultracentrifuged in a Beckman SW40 rotor at 280,000 x g for 2 hr at 4 °C. The turbid interface was collected and washed once with TS and protease inhibitors and ultracentrifuged in a Type 50 Ti rotor at 100,000 x g for 30 min at 4 °C. The resulting pellets were resuspended in the same buffer for subsequent protein quantification and ASB14 treatment.
ASB14 treatment. Membrane fractions were treated with 10% of the amidosulfobetaine nonionic detergent, ASB14, for 1 hr at room temperature with slow stirring. In Protocol I, treated membranes were ultracentrifuged in a Type 50 Ti rotor at 100,000 x g for 30 min at 4 °C. Supernatant and pellet were cleaned up using the Amersham 2D clean up kit. Before clean up, the ASB-14 pellets were resuspended in 7 M urea and 1.2 % CHAPS and ultracentrifuged to collect proteins in the supernatant. After cleaning, both protein samples, supernatants and pellets were resuspended in solubilization Buffer 1 (7 M urea, 2 M thiourea, 1.2% CHAPS and 0.4% ASB14). Protein quantification were done using a BCA kit (Pierce). For the Protocol II, ASB14 treated membranes were directly diluted in 2 vol. of solubilization Buffer 2 (7 M urea, 2 M thiourea, 4% CHAPS) and only an aliquot was cleaned up to remove interferants such us ASB14 at more than 0.5 % concentration. Protein were quantified in the cleaned aliquots.
Western blot. Proteins were separated on 7.5 % SDS-polyacrylamide gel and transfer onto a nitrocellulose membrane. Membranes 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, membranes were incubated with primary antibodies (rat monoclonal antibody anti-MRP1, Santa Cruz, 1:3000 dilution; rabbit polyclonal anti-Glut1, Santa Cruz, 1:400 dilution; affinity isolated rabbit antibody anti-actin, Sigma, 1: 1500 dilution) in blocking solution for 1 hr at room temperature, washed with TBST, three times for 5 min and incubated with the horseradish peroxidase secondary antibody in blocking solution for 1 hr at room temperature. The protein bands were visualized by the enhanced chemiluminescent method (Pierce).
Isoelectric focusing (IEF) and second-dimensional gel. IEF was carried out in 7 or 11 cm IPG´s (Bio-Rad) pH 3-10 NL. Strips were actively rehydrated in presence of sample, 5 mM TBP and 0.25 % ampholytes for 12 hr up to a total of 55 KVh and 60 KVh for the 7 and 11 cm IPG´s, respectively. 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).
MS analysis. Protein spots were manually excised and tryptic digested. Tryptic peptides were analyzed by MALDI-MS using a Micromass A@ldi™ spectrometer (Manchester, UK).

Figure 1. Preparation of membrane proteins

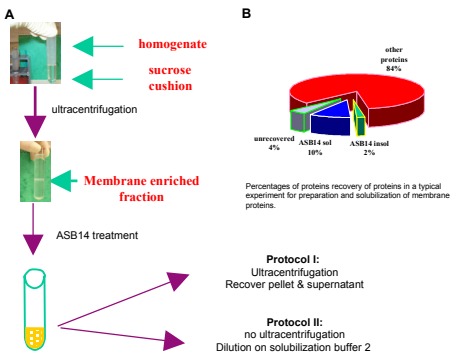

Figure 2

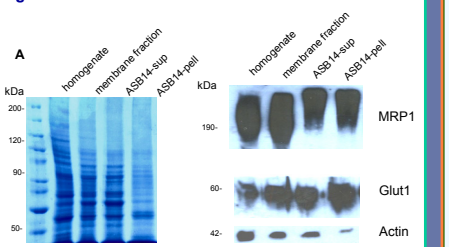

Fig. 1. A. SDS-PAGE of 15 µg proteins from J774 homogenate and membrane fraction and approximately 10 µg of proteins from ASB14 solubilized and non solubilized membrane proteins samples after the treatment of the membranes following the Protocol I (Materials and Methods). Samples were loaded in 7.5 % acrylamide gels and stained with Coomassie blue G-250. **B.** Western blot for MRP1, multiresistant related protein 1, and the Glut1, the glucose receptor 1, as markers of embedded membrane proteins. The cytoplasmic, soluble protein actin was included as a control to monitor contamination by soluble, intracellular proteins. ASB14-Sup, supernatant recovered by ultracentrifugation after treating the J774 membrane fraction with ASB14; ASB14-pell, insoluble proteins recovered by ultracentrifugation as a pellet after ASB14 treatment of the membrane fraction and resuspended in 7M urea and 1.2 % CHAPS following the Protocol I (Materials and Methods).

Figure 3

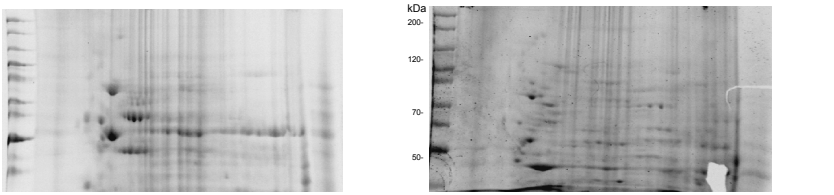

Fig. 2. 2DE separation of protein samples after the treatment of J774 membranes fractions with the nonionic detergent ABS14 following the Protocol I. 250 µg of proteins in 7M urea, 2 M thiourea, 1.2% CHAPS, 0.4% ASB14, 5 mM TBP and 0.25% ampholytes were focused in 7 cm IPG´s with 3-10 NL pH gradient. Strips were run in a Protean IEF System (BioRad) at 250 V for 30 min, 1000 V for 2 hr and 4000 V up to 55 kVh. 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) **A**. ASB14 solubilized membrane proteins; **B**. SB14 insoluble proteins recovered following protocol I.

Figure 4

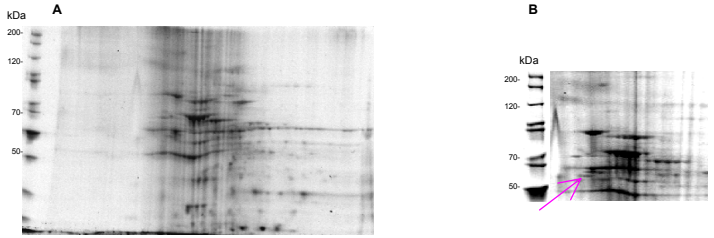

Fig. 2. 2DE separation of protein samples after the treatment of J774 membranes fractions with the nonionic detergent ABS14 following the Protocol II. 150 µg of proteins in 4M urea, 1.2 M thiourea, 2.5% CHAPS, 4% ASB14, 5 mM TBP and 0.25% ampholytes were focused in 11 cm IPG´s with 3-10 NL pH gradient. Strips were run in a Protean IEF System (BioRad) at 250 V for 30 min, 5000 V for 3 hr and 8000 V up to 60 kVh. Equilibration of strips was performed using the DTT-iodoacetamide two-step method. In the second dimension IPG´s were applied on 10 % Criterion Tris-HCl (BioRad) gels and stained with Sypro Ruby protein gel stain. Imaging was done with the Gel Doc™ documentation system (BioRad) **A**. ASB14 solubilized membrane proteins. **B**. Zoomed imaged of the protein profile from a duplicate gel in which the protein spots present improved focusing and resolution of protein isoforms when compared to samples prepared following the Protocol I. Arrow points at a protein present a low abundance with at least three well separated isoform.

RESULTS and DISCUSION

To gain insight into potential protein targets of pharmaceutical interest using a classic proteomics approach we first separated the cellular membranes and then treated them with the nonionic detergent ASB14 to extract the proteins and prepare suitable samples for 2DE separation and MS analysis. In a typical experiment following the Protocol I, 10 mg of total proteins would render after the preparation and treatment of membranes (Materials and Methods) a 10 % of proteins recovered by ultracentrifugation as a soluble supernatant and a 6 % remained as an insoluble pellet. To further analyze the proteins remaining in the ASB14-pellet, the pellet was resuspended in 7M urea and 1.2 % CHAPS, ultracentrifuged and 2 % of proteins were collected from this supernatant (Fig. 1).

The efficiency of the solubilization by the ASB14-treatment was evaluated by detecting membrane transporters showing more than ten transmembrane domains, the MRP1 and Glut1. Western blot of homogenate, membrane fraction and the post-ASB14 treatment samples, ASB14-supernatants and pellets, shows the presence of the probed transporters. Although both molecules, MRP1 and Glut1 were detected in the ASB14-supernatant there is a considerable amount of them remaining in the ASB14-pellet (Fig. 2).

In a first attempt to separate the membrane proteins extracted with ASB-14 by 2DE, proteins in the ASB14-supernatants and pellets were prepared using the solubilization Buffer 1 (Protocol I, Materials and Methods). 2DE minigels showed few well focus spots and rather poor resolution with vertical streaking (Fig. 3, A and B). Therefore, to improve the quality and efficiency of the separation, the protocol was modified as follow. We scale up the relative focusing power going from 7 cm 11 cm IPG strips. To decrease the streaking effects, possible due to the presence of traces of interfering compounds used for the precipitation and washing of the proteins during the clean up step, we diluted the ASB14-treated membranes with solubilization Buffer 2 (Protocol II, Materials and Methods) and used for electrofocusing without previous clean up. For the quantification of the proteins small aliquots were subjected to clean up since ASB14 interfere with known protein quantification methods. As shown in Fig. 4, A and B, the Protocol II rendered improved 2DE profiles, an increased number of proteins spot with a greatly better focusing and superior resolution of protein isoforms. Additionally, we achieved highly reproducible profile separations with independent membrane preparations.

Furthermore, major different proteins stops were excised and identified by MALDI MS indicating that the protocol is compatible with downstream mass spectrometry methods for identification of proteins.