Combination of ProMetHEUS™ FFE with SDS-PAGE for the two-dimensional separation of mitochondrial membrane proteins

Introduction

There exist a variety of well-established techniques for the separation of soluble proteins like two-dimensional electrophoresis (2-DE) and chromatographic approaches like reversed phase high performance liquid chromatography (RP-HPLC) or ion exchange chromatography (IEC).

The separation and analysis of membrane proteins, however, still remains a difficult task, especially in the context of proteomics research. Regarding chromatographic approaches, the poor solubility as well as the extreme hydrophobicity of the integral membrane proteins directly interferes with successful separations. Not to mention quantitative aspects and the problems encountered with larger proteins.

Despite intense efforts for the development of new detergents, reducing agents and other additives, classical 2-DE of integral membrane proteins generally suffers from significant precipitation effects during sample application, isoelectric sample focusing and sample transfer from the first to the second dimension. This hampers or even prevents the global characterization of the membrane proteome of cells and/or organelles.

Liquid-phase isoelectric focusing (IEF) by free-flow electrophoresis (FFE) circumvents the sample precipitation phenomena encountered during the phase transfers into and out off the IEF-gel, because sample buffer and separation buffer are pretty much identical. Precipitation phenomena during the IEF itself can be addressed by the addition of neutral and zwitterionic detergents. Furthermore, the transparent front plate of the separation chamber permits to continuously monitor the formation of precipitations during an IEF-run, thus allowing the optimization of the separation conditions very efficiently. In addition, the continuous operation principle of FFE enables the analysis of low abundant proteins (membrane proteins typically have lower abundances) by the processing of large sample volumes including subsequent fraction concentration. Last but not least, FFE can be combined with virtually any other separation technique to increase the resolution even further. This is based on the fact that the fractionated samples are still in solution after FFE-processing. For the present application note FFE was combined with SDS-PAGE, another excellent technique for the separation of membrane proteins [Dunn & Bradd]. The capabilities of this new combined approach are exemplified by the 2-D separation of mitochondrial membrane proteins including subsequent identification by MALDI-MS.

Material and Methods

Chemicals

Standard chemicals for electrophoretic separations and mass spectrometry were from Aldrich (Taufkirchen, Germany), Fluka (Taufkirchen, Germany), Serva (Heidelberg, Germany), Sigma (Taufkirchen, Germany) and VWR

international (Darmstadt, Germany). Trypsin was purchased from Roche Molecular Biochemicals (Mannheim, Germany). ProLyte separation media including Prolytes™ were from Tecan (Männedorf, Switzerland).

Preparation of membrane protein sample

1 mL of crude mitochondria (7.5 mg/mL) were prepared according to [Zischka et al., 2003]. Subsequently they were centrifuged for 30 min (25000 g, 4°C). Then, the pellet was treated with 1 mL of 100 mM Na_2CO_3 for 30 min on ice. After ultracentrifugation (100000 g, 4°C, 30 min) the pellet was washed with 1 mL of 100 mM Na_2CO_3 and ultracentrifuged again (100000 g, 4°C, 30 min). Finally, the pellet was lysed with 1 mL of FFE-separation media (see below) and centrifuged once more (25000 g, 18°C, 30 min). The supernatant corresponded to 1 mL of mitochondrial membrane proteins having a concentration of 3.4 mg/mL.

2-D-fractionation of membrane proteins

Free-flow IEF separation was performed using a FFE instrument. For detailed information on FFE please refer to [Krivankova & Bocek, 1998]. In short, proteins are deflected and focused in an electric field based on their pH-dependant charge density. This is realized by the continuous transport of the sample in a thin, laminar, pH-graded buffer flow and the perpendicular application of an electric field as a deflecting force.

The mitochondrial membrane proteins were subjected to the FFE separation using the following settings: (i) The anodic and cathodic circuit electrolytes consisted of 100 mM H_2SO_4 and 100 mM NaOH, respectively. (ii) The anodic and the cathodic electrolyte stabilizer (media inlet 1 and 6/7) was 7M urea, 2M thiourea, 7% glycerol, 0.03% hydroxypropylmethylcellulose (HPMC) as well as 100 mM H_2SO_4 and 100 mM NaOH, respectively. (iii) The separation media (media inlet 2-5) consisted of 7M urea, 2M thiourea, 7% glycerol, 0.03% HPMC as well as 10 mM DTT and 0.1% Triton X-100. Prolytes™ were used to establish a linear pH-gradient from 3-11.5. (iv) Counterflow media consisted of 7M urea, 2M thiourea, 7% glycerol and 0.03% HPMC.

FFE was performed in horizontal mode at 10°C with a total flow rate of ~56 g/h within the separation chamber at a voltage of 820 V (~ 19 mA). The sample was applied to the separation chamber with a flow rate of 0.9 mL/h via the middle sample inlet. Residence time in the separation chamber was ~21 min. Fractions were collected in 96-well plates (A1, B1, C1, … = anode end, …, F12, G12, H12 = cathode end). Subsequently, second dimension separation of the FFE fractions was done by SDS-PAGE using an XCell SureLock™ Mini-Cell (Invitrogen, Carlsbad, CA, USA) in combination with precast NuPAGE® 4-12% Bis-Tris gels (Invitrogen). Staining of the proteins was carried out using a SilverQuest™ kit (Invitrogen) according to the manufacturer's instructions.

Fraction concentration and electrophoretic separation

Based on the SDS-PAGE analysis a set of FFE fractions was selected for detailed protein identification. The fractions were concentrated approximately 30-40fold by means of Vivaspin 6 centrifugal concentrators (Vivascience, Hannover, Germany) according to the manufacturer's instructions. The concentrators were equipped with polyethersulfone membranes having a cut-off of 5 kDa.

Subsequently, the enriched FFE fractions were analyzed by SDS-PAGE according to the procedure mentioned above. Staining of the proteins was carried out using Coomassie® brilliant blue G 250.

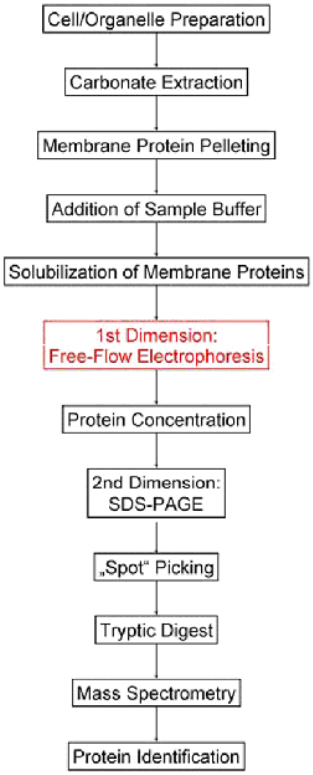
Protease treatment of protein-containing gel pieces and MALDI-MS analysis

All protein bands visualized by Coomassie® staining were cut out from the respective gels and a sequence dependent protease treatment was performed using a ProTeam™ Digest instrument (Tecan). The reactions and subsequent MS-analyses were carried out according to [Zischka et al., 2003]. Briefly, gel plugs were washed/equilibrated with ammonium bicarbonate in acetonitrile, and digested using trypsin. The reaction was terminated by trifluoroacetic acid. Peptides were purified utilizing the ZipTip® (Millipore, Billerica, MA, USA) technology. Purified peptides were eluted directly onto the MALDI target plate using á-hydroxy-cyano-cinnamic acid as a matrix. MALDI-MS analysis was performed on an Autoflex® instrument (Bruker Daltonics, Billerica, MA, USA). Calibrated and annotated spectra were subject to a database search utilizing Bruker's BioTools 2.0 and the Mascot 1.7 search engine.

Results and Discussion

Analysis workflow

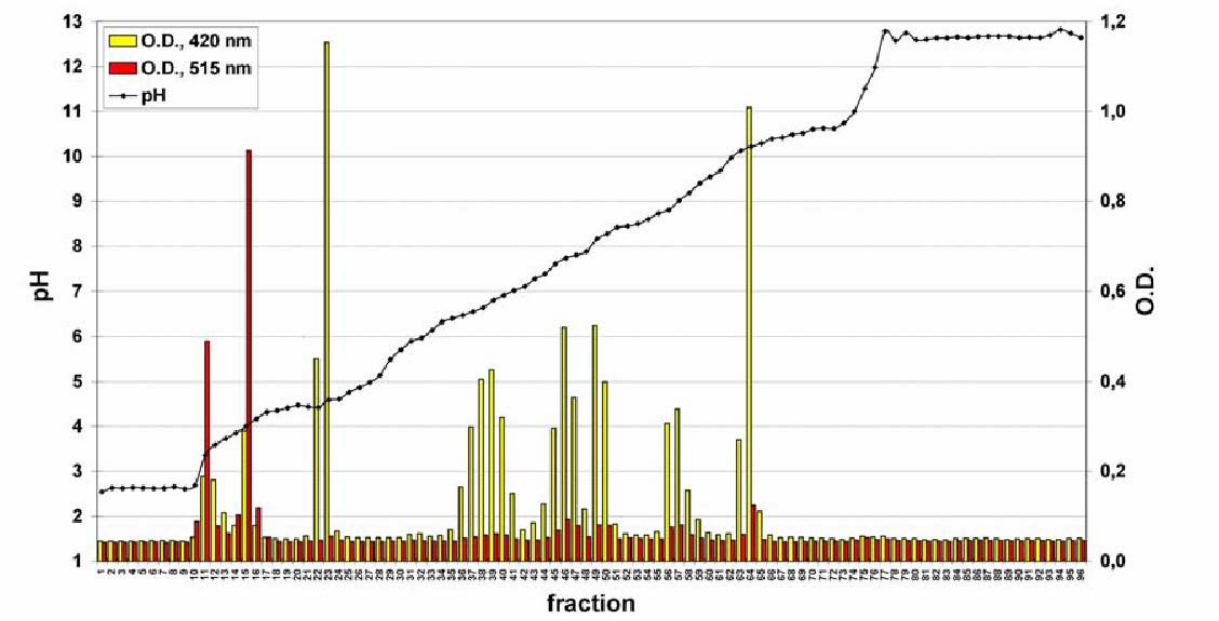
A classical workflow for the one-dimensional separation of membrane proteins is based on carbonate extraction for sample preparation, solubilization of the hydrophobic proteins by urea/thiourea/detergent-containing sample buffers, separation of the proteins by SDS-PAGE and identification of the proteins by tryptic digest and MS. To complement and enhance this approach we integrated a FFE IEF separation dimension followed by ultrafiltration to concentrate the fractions:



Typical workflow for the FFE-based two-dimensional separation of membrane proteins

Free-flow electrophoresis

The IEF-separation of membrane proteins ideally should take place in a buffer system that matches the solubilization buffers which are typically used for membrane proteins. Thus, we used additives like 7M urea, 2M thiourea, Triton X-100 as a detergent as well as DTT to provide reducing conditions. Substitution of Triton X-100 by other non-ionic detergents like Triton X-114, n-Octyl-ß-D-glucopyranoside, n-Dodecyl-ß-D-maltoside or zwitterionic detergents would have been possible, too. The use of additives slightly decreases the resolution of an IEF-FFE experiment (if compared to native separations), but just to a minor extent: The pH-gradient as well as the distribution of test-pI-markers were still fine (see top figure, next page).



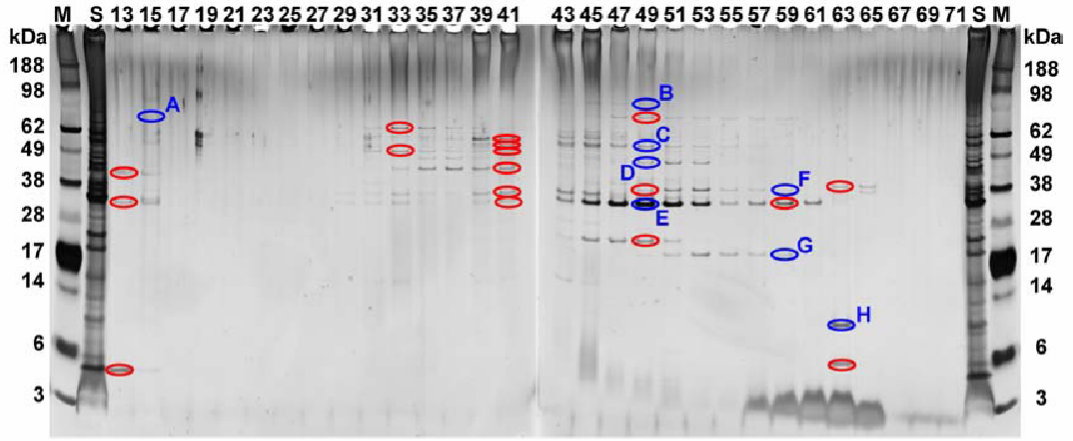
pH-gradient in the separation chamber and the 96 fraction wells, respectively, as well as isoelectric focusing of six yellow and two red IEF-markers to demonstrate the performance of the Pro Team™ FFE instrument under the conditions used for the separation of membrane proteins.

The analytical SDS-PAGE gel after the IEF-FFE run (see middle figure, next page) showed a lot of protein bands that just occur in one or two lanes. This indicates the high resolution of the FFE fractionation. The relatively broad distribution of other bands could either be explained by the existence of several isoforms having different pI-values or the presence of different proteins with similar molecular weights. In some cases, the hydrophobic nature of membrane proteins might have lead to strong protein-protein interactions that persisted even in the presence of urea, thiourea and detergent. Nevertheless, the broad distribution of the proteins over a pH-range from 3.5-10.5 in combination with almost no visible overlap of the protein bands and a minimal background allowed efficient “spot”-picking of the bands. Beforehand, the amount of proteins to be loaded onto the gel was increased 30-40fold by concentration of selected fractions with ultrafiltration.

Protein identification

The protein identification by tryptic digest of excised bands (ellipses on SDS-PAGE) and subsequent MALDI-MS analysis yielded exclusively mitochondrial proteins being located within or close to the inner or outer mitochondrial

membrane (see table on bottom, next page). Solely, the location of protein "B" could not be assigned in detail. The prediction of the transmembrane domains of putative protein "H" by TMpred (Hofmann & Stoffel, 1993) resulted in two regions which suggests that it represents a membrane protein, too. The excellent correlation between the theoretical molecular weights of the identified proteins and the positions of the analogous bands on the SDS-PAGE gel further strengthened the reliability of the assignments.



SDS-PAGE of individual FFE fractions. M = MW-marker; S = crude sample; 13-71 = fraction numbers; blue ellipses = analyzed and identified proteins; red ellipses = analyzed, but unidentified proteins.

	ID	Name	Score	Seq Cov	Location	MW
A	P07213	Mitochondrial precursor proteins import receptor	95	30%	mom	70123
B	P19414	Mitochondrial aconitate hydratase (precursor)	153	33%	mit	85368
C	P07251	Mitochondrial ATP synthase α chain (precursor)	128	49%	mm	58618
D	P16547	Mitochondrial outer membrane 45 kDa protein	168	50%	mom	44580
E	P04840	Outer mitochondrial membrane protein porin 1	78	28%	mom	30429
F	P36060	NADH-cytochrome b5 reductase (precursor)	111	56%	mom/ims	34138
G	P30902	Mitochondrial ATP synthase D chain	150	83%	mm	19678
H	(Z46260)	Putative protein	52	62%	unknown	12061

Identified proteins corresponding to blue ellipses on previous SDS-PAGE. mom = mitochondrial outer membrane; mm = mitochondrial membranes; mit = mitochondrial; ims = inter membrane space.

The fact, that more than 50% of the excised bands could not be assigned (red ellipses on SDS-PAGE) was based on several reasons. First of all, the digestion of membrane proteins by proteases is not as efficient as the digestion of soluble proteins. This is due to the lack of charged residues like Arg and Lys for trypsin inside the transmembrane domains, as well as steric and hydrophobic hindrance of the proteolysis reaction itself. Thus, the number of analyzable peptides is greatly reduced which prevents the identification of membrane proteins – at least by classical MALDI-MS. The lack of a possibility to run MS-MS-experiments has to be taken into account, too. Not to mention the low staining intensity of several protein bands, i.e. the low abundance of these proteins which further decreased the probability to identify the proteins.

Conclusion

It is extremely difficult and laborious to analyze membrane proteins based on existing approaches. Exemplified by the fractionation of mitochondrial membrane proteins, Free-Flow Electrophoresis can greatly improve the separation of membrane proteins, thus laying the foundation for a much more efficient identification. The combination of the method with MS-MS-techniques should further improve the sensitivity to allow the analysis of even more membrane protein species.

References

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