CHAPTER 2

TWO-DIMENSIONAL BAC/SDS-PAGE FOR MEMBRANE PROTEOMICS

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Abstract: Although often used in membrane proteome studies, conventional two-dimensional gel electrophoresis (2-DE) is not well suited for resolving hydrophobic proteins. Nevertheless, an alternative technique, two-dimensional BAC/SDS-PAGE (2-DB) using the cationic detergent benzyldimethyl-*n*-hexadecylammonium chloride (BAC) in the first and the anionic detergent SDS in the second dimension can be utilized as a powerful tool for the separation and analysis of membrane proteins. Systematic studies demonstrated the advantage of 2-DB over one-dimensional SDS-PAGE and 2-DE with regard to membrane proteomics. While in 2-DE gels, in particular proteins with more than one transmembrane domain (TMD) are underrepresented, one-dimensional SDS-PAGE lacks sufficient resolution for large scale analyses. In contrast, 2-DB enabled the identification of extremely hydrophobic proteins like cytochrome-c oxidase subunit I from *S. cerevisiae* with a total of 12 known TMD. Especially the application of tube gels in the first dimension as well as the recent introduction of improved buffer systems hold a great potential for future 2-DB-based membrane studies.

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1. INTRODUCTION

The separation of membrane proteins via conventional two-dimensional gel electrophoresis (2-DE) consisting of a first dimension isoelectric focussing step and a subsequent second dimension SDS-PAGE is, although still widely used, strongly biased against hydrophobic proteins. One the one hand, IEF is limited to the usage of zwitterionic or non-ionic detergents, providing weak solubilizing capabilities with regard to hydrophobic proteins in contrast to strong ionic detergents, for example SDS. On the other hand, proteins tend to precipitate upon reaching their isoelectric points (pI) or during the transfer to the second dimension – this is particularly true for membrane proteins. Furthermore, membrane proteins often have pIs in the alkaline region, which in 2-DE is generally characterized by inferior resolution.

Although protocols have been constantly improved in recent years (Molloy 2000; Olsson et al. 2002; Luche et al. 2003), hydrophobic membrane proteins, especially those with multiple transmembrane domains (TMD) are generally underrepresented in 2-DE based proteome studies (Santoni et al. 2000). To account for these inherent limitations alternative electrophoretic techniques have to be applied.

While common one-dimensional SDS-PAGE is a powerful tool for separating hydrophobic membrane proteins (Reinders et al. 2006a), it only provides minor resolution of complex protein samples, and consequently is not suited for differential analyses.

However, alternative 2-DE methodologies, for instance combining SDS-PAGE with PAGE systems based on the usage of cationic detergents like benzyldimethyl-*n*-hexadecylammonium chloride (BAC) (Macfarlane 1983, 1989; Hartinger et al. 1996) or cetyltrimethylammonium bromide (CTAB) (Buxbaum 2003), enabling two-dimensional separation of hydrophobic membrane proteins, have gained more attention in recent years. Thereby, extremely hydrophobic membrane proteins with multiple TMD can be separated with relatively high resolution for subsequent identification by mass spectrometry.

2. TWO-DIMENSIONAL BAC/SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

First introduced by Macfarlane et al. for the separation of base labile protein methylation (Macfarlane, 1983), BAC-PAGE was then improved towards a two-dimensional technique by combination with a subsequent second dimension SDS-PAGE (Macfarlane, 1989). Although, both dimensions comprise a separation according to the molecular weight, slight differences in protein migration properties within the two systems lead to an enhanced resolution, resulting in an elliptical separation area. Already in 1996, Hartinger et al. demonstrated the potential of combined two-dimensional BAC/SDS-PAGE (2-DB) for the separation of membrane proteins (Hartinger et al. 1996). The second dimension SDS-PAGE provides full compatibility with downstream methods like Western Blotting, a broad variety of staining

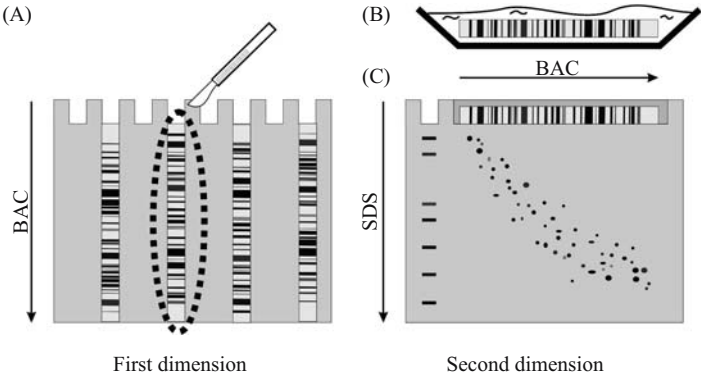

Figure 1. Scheme of a two-dimensional BAC/SDS PAGE using slab gels. (A) Protein samples are separated on the first dimension BAC-PAGE. After visualization of proteins, an entire gel lane is excised. (B) The excised lane is re-buffered in 100 mM Tris, pH 6.8 for 30 min and afterwards incubated in 3× SDS sample buffer for another 5-10 min in order to exchange BAC for SDS. (C) The gel lane is transferred onto the second dimension SDS gel and fixed with a hot agarose solution. After separation staining reveals a characteristic spot pattern within an elliptical area.

techniques and mass spectrometric analysis. Therefore, in recent years several studies have revealed the great potential of this alternative technique for membrane proteomics (Otto et al. 2001; Daub et al. 2002; Diao et al. 2003; Godl et al. 2003; Coughenour et al. 2004; Zahedi et al. 2006).

3. GENERAL WORKFLOW

The general scheme of 2-DB is depicted in Figure 1: Samples are first separated towards the cathode in an acidic PAGE system based on the cationic detergent BAC. Afterwards, protein lanes can be visualized by direct immersion into a colloidal Coomassie staining solution. However, since Coomassie tends to precipitate in presence of cationic detergents which in turn leads to enhanced background staining, an intermediate washing step of at least 2 h is recommended in order to remove BAC from the gel surface.

After visualization whole protein lanes are excised and prepared for the second dimension. Since SDS-PAGE utilizes a more alkaline buffer system than BAC-PAGE, first of all gel lanes have to be re-buffered. Afterwards, BAC is exchanged for SDS by incubation in 3× SDS sample buffer. Finally, protein lanes are transferred onto a second dimension gel for separation and fixed by a hot agarose solution.

4. IMPROVEMENTS

While less complex samples can generally be separated using small gel sizes (approximately 7 × 7 cm) in both dimensions, for samples of higher complexity switching

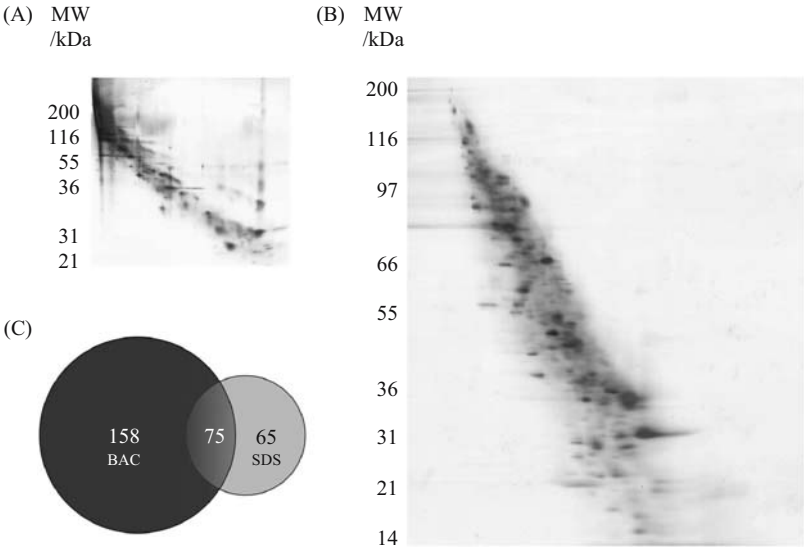

Figure 2. Two-dimensional BAC/SDS-PAGE of platelet membrane proteins. While not only large gels are recommended for complex samples, utilizing tube gels for the first dimension furthermore provides better resolution and more efficient transfer of proteins to the second dimension (less vertical smearing). (A) Small slab gel in the first dimension (7 × 7 cm). (B) Large tube gel in the first dimension (1 mm × 15 cm). (C) Summary of the human platelet membrane proteome study (Moebius et al. 2005). 158 proteins were exclusively identified from 2-DB gels, 65 from 1D-PAGE. An overlap of 75 proteins was identified from both types of gels.

to larger high resolution gels is recommended (Figure 2). Here, slab gels as well as tube gels can be utilized. However, the usage of slab gels is much more complicated in terms of handling (Figure 3).

In general, first dimension BAC-PAGE requires higher voltages and prolonged running times than SDS-PAGE, resulting in an increased heat development – especially in case of large dimension slab gels. Therefore, cooling of running buffers during separation is mandatory.

Besides, an incomplete transfer of proteins from the first to the second dimension can be noticed for slab gels. The usage of tube gels with inner diameters of 1 mm and less totally abolishes this limitation and furthermore provides an improved resolution leading to a higher number of identifications in proteome studies. For this reason, the usage of tube gels is essential for differential studies. Moreover, after first dimension separation, time-consuming staining procedures which might be accompanied by loss of material can be omitted, as the entire gel can be transferred to the second dimension without the need for prior excision.

Resulting from our experience in the separation of a broad variety of samples with 2-DB, resolution strongly depends on the nature of the separated sample. Furthermore, upon long separation times, band broadening during first dimension separation can be observed.

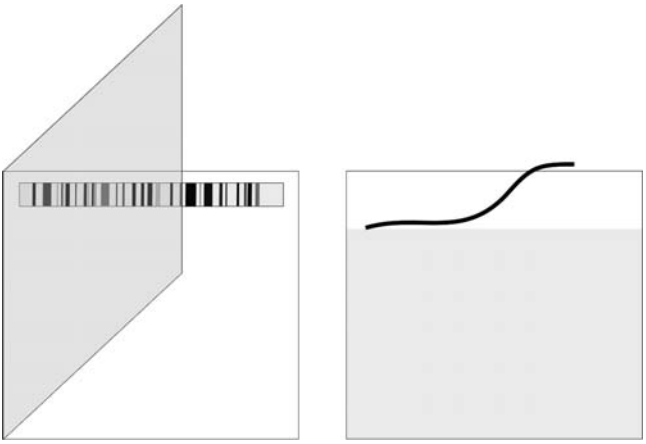

Figure 3. Processing of large slab (left) and tube (right) gels. Since transferring whole gel lanes of approximately 15–20 cm length onto polymerized second dimension gels mostly results in extensive damage of the gel, a more complicated but also safer strategy is recommended here. First the excised gel lane is placed and fixed between two glass plates. Then the separation gel is poured beneath the lane. Finally, a stacking gel is poured on top, enclosing the first dimension gel lane. In case of tube gels, the gel is transferred onto the already polymerized second dimension separation gel and fixed by hot agarose solution.

In general, after membrane purification, it is highly recommended to introduce additional steps like carbonate- (Fujiki et al. 1982) and/or Triton X114-extraction prior to electrophoresis in order to reduce the amount of contaminating soluble proteins. Especially when separating plasma membrane enriched samples, resolution may be impaired in both dimensions. In that case protein precipitation prior to 2-DB may result in an improved separation, however it has to be kept in mind that precipitation procedures are generally not quantitative and might lead to unspecific loss of material.

Recently, an improved BAC-PAGE protocol was introduced, particularly compensating for the inferior efficiency of the stacking gel when compared to common SDS-PAGE systems (Kramer 2006). By systematic studies the composition of gel buffers, running buffers as well as the sample buffer were optimized, resulting in a higher resolution and shorter separation time, comparable to SDS-PAGE (Kramer 2006). Although the impact of these improvements was only investigated for one-dimensional BAC-PAGE, they nevertheless hold a great potential for 2-DB as well. However, this remains to be demonstrated in future studies.

5. POTENTIAL FOR MEMBRANE PROTEOME STUDIES

In systematic studies we demonstrated the potential of 2-DB compared to 2-DE (Zahedi et al. 2005) and SDS-PAGE (Moebius et al. 2005) regarding the separation of membrane proteins.

While 2-DE separation of purified endoplasmatic reticulum membranes from *Canis familiaris* yielded only a few spots after visualization by silver staining, an unequal

higher amount of protein spots could be visualized after 2-DB. Among them, Sec61α with a total of ten known TMD and a grand average hydrophobicity (GRAVY) index of 0.558 could be identified by Western blotting as well as mass spectrometry. Furthermore, 54 distinct ribosomal proteins were identified, which cannot be sufficiently resolved by 2-DE since their pIs range from 9 to 12. Furthermore, the separation of mitochondrial membranes from *Saccharomyces cerevisiae* yielded the subsequent identification of the extremely hydrophobic cytochrome-c oxidase subunit I with a total of 12 known TMD and a GRAVY index of 0.74 by mass spectrometry (MS).

In a comprehensive study of the human platelet membrane proteome we demonstrated the need for a combined application of 2-DB and one-dimensional SDS-PAGE for maximizing the amount of identified proteins. Since both techniques address different subproteomes (Figure 2C) they may be utilized in a complementary way. In 2-DB, a higher resolution increases the local protein concentration and facilitates identification. However, in SDS-PAGE, whole lanes can be cut into equidistant slices, eliminating the need for protein visualization prior to excision. Thereby, even proteins, which cannot be visualized by staining procedures and therefore will escape detection after 2-DB, can be identified by subsequent MS.

6. COMPARISON TO OTHER TECHNIQUES

Another two-dimensional PAGE technique, doubled SDS (dSDS), introduced by Rais et al. (2004), is capable of resolving hydrophobic proteins with GRAVY indices of up to 0.86 – however it is has a lower resolution compared to 2-DB due to a smaller accessible separation area.

Since due to the lower resolution in 2-DB gels mostly several proteins co-localize within a single spot, in contrast to conventional 2-DE the usage of LC-MS/MS for protein identification is recommended instead of MALDI-MS.

Table 1 summarizes advantages and properties of the presented electrophoretic methods regarding (membrane) proteome studies.

7. OUTLOOK

Two-dimensional BAC/SDS polyacrylamide gel electrophoresis has been established as further tool in the field of proteome research, especially regarding the separation and analysis of membrane proteins. It is by far more efficient in resolving membrane proteins than common 2-DE and furthermore can be utilized in a complementary way to one-dimensional SDS-PAGE. Therefore, among other techniques, future proteome studies focussing on membrane proteins should include 2-DB as well.

Despite its lower resolution compared to 2-DE, 2-DB can also be applied in combination with the difference gel electrophoresis technique (DIGE) (Unlu et al. 1997; Reinders et al. 2006b), enabling the highly reproducible differential analysis of biological membrane samples.

However, for complex sample mixtures DIGE requires a very high resolution and distinct protein spots. Although the afore mentioned improvements by Kramer further

Table 1. Comparison of different PAGE methods

	1D-PAGE	2-DE	2-DB	dSDS
Resolution	–	++	o	O
Differential studies	–	++	+	O
Proteins are focussed to	Bands	Spots	Spots	Spots
Local concentration	Low	High	High	High
Separation area	Small lane	Entire gel	Elliptical area	Elliptical area
Isoforms	–	+	–	–
Membrane proteins	+	–	+	+
Alkaline proteins	+	–	+	+
Visualization before MS	Not necessary	Necessary	Necessary	Necessary
Amenability to analysis	Entire lane	Only visualized spots	Only visualized spots	Only visualized spots
Recommended MS strategy	LC-MS/MS	MALDI-MS	LC-MS/MS	LC-MS/MS

enhance resolution during first dimension BAC-PAGE, additional prefractionation of complex mixtures might be necessary in order to account for the high demands of differential gel electrophoresis.

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