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# Blue Native Polyacrylamide Gel Electrophoresis – A Functional Approach To Plant Plasma Membrane Proteome Studies

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Licentiate thesis in plant biology

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2004

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# ANALYSIS OF PROTEINS AND PROTEOMES

## Four different levels of global cell biology studies

The breakthrough of molecular biology and its wide applications made it possible to create whole new fields in science such as genomics, transcriptomics and proteomics. The first complete genome published was that of *Haemophilus influenzae* (Fleischmann et al., 1995). The human genome sequence was completed and published in 2001 (Venter et al., 2001), and was the project that made the non-scientific public aware of sequencing projects. In parallel, other genome projects of the same importance, though not with the same public attention, were conducted. For instance, the complete genome of the model plant *Arabidopsis thaliana* was fully sequenced by the Arabidopsis Genome Initiative, published in the year 2000. Other systematic sequencing projects include sequencing the *Oryza sativa* genome. The number of genes found in different plant species vary, but more than 80 % of the predicted genes in *A. thaliana*, which has a small genome by plant standards, have homologues in rice (Yu et al., 2002; Goff et al., 2002), indicating that the number of functional units in the cells may not differ significantly. The distribution of proteins into predicted functional classes has a similar pattern when *A. thaliana* and rice are compared (Yu et al., 2002). Therefore, some conclusions may be drawn between species with data obtained from only one species that is compared to another, fully sequenced, species.

The sequencing projects have led to the creation of publicly available databases of genes and expressed sequenced tags (ESTs). An EST is partial cDNA derived from extracted mRNA where no knowledge of the function of the original mRNA necessarily exists, and is useful in the process of mapping transcription. The research community has benefitted from the sequencing projects, enabling research projects that otherwise would not have been possible. However, sequencing projects and EST databases are not aimed at explaining the function of the gene products. Thus, sequence databases rely on sequence similarity to indicate gene product function and cell location and therefore, though useful, the information may not always be fully correct or give a complete description of function and location of gene products.

A functional genomics approach is obtained when transcriptomes (total mRNA) are studied from differently treated material (Seki et al., 2001). Other methods, such as T-DNA insertions to create knockout mutants, have been employed to address functional questions (Goujon et al., 2003; Hartung et al., 2002). This T-DNA based approach may give information as to the function of gene products, provided that the gene is not essential for vitality.

Protein expression studies was the next level of global investigation to be implemented. In this approach, a tissue is examined and the proteome, i.e., the protein content profile, of the tissue is studied, as in Santoni et al. (1999a). The approach is sometimes called proteomics, though some caution may be in place as the studies often are not fully systematic in their nature but rather study a particular material at a particular time point in a particular environment. From here on I will use the term “proteome study” to denote

any protein content profile study, including the content profiles of subproteomes, as the term “proteome study” better describes what is studied. Most proteome studies describe the presence of polypeptides and do not have the capacity to give indications of function or functional arrangement of the polypeptides.

Yet a step further in the list of “-omics” is the study of the metabolome, i.e. global metabolite levels, which could be explained as the metabolite phenotype (Sumner et al., 2003). The combined volume of the data from these four levels – genomics, transcriptomics, proteomics and metabolomics – should provide a powerful tool to understand how life is physiologically possible in the cell. This thesis will focus on a functional aspect of proteome studies by investigating functional polypeptide arrangement in the plant plasma membrane.

### **Electrophoretic methods are important tools in proteome studies**

Proteome studies have been conducted for almost thirty years (O'Farrell, 1975) but in the last years the techniques to identify proteins have made great progress. A majority of the studies have had an emphasis on the singular polypeptides rather than on the functional protein complexes. Crude total protein extraction followed by solubilisation has resolved up to 3600 proteins from leaf tissue (Giavalesco et al., 2003), though the resolution when analyses are done may be 5-75 % of the reported number of proteins (Pietrogrande et al., 2003), depending on overlapping protein spots and on what is actually put into the term resolution.

Electrophoresis, such as SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and IEF/SDS-PAGE (isoelectric focussing, i.e. migration of polypeptides in a pH gradient, until the polypeptides are focussed to their isoelectric point, followed by SDS-PAGE in a second dimension), has proven itself a useful separation tool for protein studies. Proteins are inherently charged and their charge may be enhanced by binding components with various binding and electrophoretic properties, such as sodium dodecyl sulphate (SDS). Electrophoresis has a great combinatorial strength as the accompanying analysis methods possible include dyes, western blotting and mass spectrometry. Different approaches of electrophoresis may be employed, depending on whether a great amount of protein – where methods like two-dimensional gel electrophoresis followed by dye or silver staining may be suitable (Gygi et al., 2000) – or a small amount of protein is to be analysed – where methods like capillary electrophoresis using UV-detection, as described in Shen & Smith (2002), may be used. In this thesis, only abundant proteins are studied and thus there is no immediate need for more sensitive methods than Coomassie staining.

Typically, cytosolic proteins are easily separated using electrophoresis. Integral membrane proteins are more difficult to separate. For proteomic studies, this may cause a problem, as transmembrane proteins can comprise approximately 25 % of the total gene product population, as predicted in genomic studies of several sequenced organisms (Jones, 1998). Thus, there is a need for alternative tools when studying hydrophobic proteomes.

Gel electrophoresis requires no prior fractionation, though prefractionation into subproteomes of different kinds improves the resolution possibilities of what is being studied (Cánovas et al., 2004). Prefractionation may include the purification of a particular

organelle or fractionation of components from a particular tissue by methods such as aqueous two-phase partitioning (Larsson et al., 1987), sucrose gradient fractionation (Navarre et al., 2002; Turner et al., 1998), size-exclusion chromatography (Sazanov et al., 1998) or organic solvent fractionation (Seigneurin-Berny et al., 1999).

IEF/SDS-PAGE is presently the standard method for proteome studies, though has its disadvantages such as an inability of resolving hydrophobic proteins well (Santoni et al., 1999a). Where IEF/SDS-PAGE may have a resolution of a thousand or a couple of thousand components (Pietrogrande et al., 2002; Shen & Smith, 2002), alternative separation techniques such as capillary electrophoresis could increase the level of separation to a few hundred thousand components (Simó et al., 2002). This implies that we may see new and more efficient approaches as well as combinations of approaches for proteomic studies in the future as new techniques are explored. As yet, however, capillary electrophoresis is used in metabolomics approaches, mainly as a prefractionation alternative to liquid chromatography or gas chromatography prior to mass spectrometry (Weckwerdt, 2003). The dominating approach in proteomics is still using the well established two-dimensional gel electrophoretic methodology. Another approach that could be used is size-exclusion chromatography, but presently the limitations on this method by hydrophobic and electrostatic interactions of developed systems make it difficult to apply on native proteins (Stulík et al., 2003), so two-dimensional electrophoresis is still the method of choice for most proteome studies.

### **Methodologies for proteome and protein interaction studies**

A trend in current proteomics research is to introduce more dimensions in separation in order to extract new sets of information (Marko-Varga et al., 2003). Here, the concept “dimension” is to be understood as a method that adds a new type of resolution potential. An example is IEF/SDS-PAGE, where the first dimension separates according to isoelectric point and the second dimension separates according to denatured molecular mass. However, IEF/SDS-PAGE is inherently incapable of giving information on most protein interactions.

An approach not uncommon in addressing functional issues as protein-protein interactions is the yeast two-hybrid system approach. In this technology, a protein fused to another protein with a DNA-binding domain is created and transformed into yeast, together with a protein fused to a transcription activator of a protein with a known assay present in the host. If the two studied proteins – the fusion proteins – interact, the transcription activator ensures that the assayed protein is transcribed (Causier & Davies, 2002). Interaction by this baiting approach is an indication of possible *in vivo* interaction. A strength of the method is that any protein pair that can be fused to the expression system components can be studied and therefore it is useful for low copy number interaction studies such as signalling pathways. A weakness is that the technology requires some initial knowledge of which protein interaction candidates to study (i.e., to choose a bait), and that false positives may be abundant (Causier & Davies, 2002). Further, the methodology is useful only under the assumptions that only two components interact or that their interaction is not affected by a third component interaction. The technology, however, is widely used and considered reliable.

An interesting approach to proteome studies was recently made, as Krijgsveld et al. (2003) fed organisms with labelled nutrition ( $^{15}\text{N}$ ), then studied the proteome with respect to this label, using IEF/SDS-PAGE. This shows synthesis profiles of the proteome as well as protein content and applied systematically, the approach may give an indication of protein turnover rates that enables whole-proteome studies on the turnover, i.e., a temporal dimension. Another IEF/SDS-PAGE-based method is a comparative approach where different dyes label samples that are pooled and differences between the original pools can then be distinguished on a single gel (Tonge et al., 2001).

An alternative way to deal with the issue of extracting information from proteomes using several dimensions of separation was size-exclusion chromatography followed by HPLC (Lecchi et al., 2003). The approach is promising, but requires further development until it can compete with IEF/SDS-PAGE.

### **It is often desirable to study subproteomes rather than total proteomes**

To avoid too complex mixtures to analyse in proteome studies, subproteomes are useful (Cánovas et al., 2004). The presence of a subproteome, such as an organellar membrane, is confirmed using a marker in the subproteome. There are several markers widely accepted for membranes and organelles. Not all subproteomes need to be organellar subproteomes; proteomic studies may be conducted on other protein entities such as the photosystems, PSI (Zolla et al., 2002) or PSII (Zolla et al., 2003).

Protein localisation in the cell is often due to a targeting sequence in the newly synthesised polypeptide. Theoretical prediction methods have been developed for predicting in which subproteome certain membrane proteins end up due to cell sorting information in targeting sequences. Several of these methods appear to work well at least for groups of proteins, but the models have mainly been developed for mitochondrial and chloroplast proteins and they are yet to be optimised (Emanuelsson & von Heijne, 2001). The prediction methods are not entirely without error margin. In *A. thaliana*, a chloroplastic protease complex contained some polypeptide components predicted to be mitochondrial (Peltier et al., 2001) and IEF/PAGE proteome studies of mitochondria from *A. thaliana* contained many proteins that according to the prediction methods had chloroplast localisation (Millar et al., 2001). To the best of my knowledge, no method is yet developed for predicting plasma membrane localisation.

### **Different identification approaches**

Molecular mass can be used as a means of identification, and may be measured in more or less elaborate ways. The least detailed measurement is perhaps according to electrophoretic migration of the proteins. As the migration characteristics of electrophoresed proteins depend on both their hydrophobicity, size, shape and charge, this may give misleading indications as to the nature of their true molecular mass if the characteristics of the analysed proteins differ from the those of the standard proteins used. Another approach to determine molecular mass is sedimentation velocity, where the molecular mass of the protein may be calculated from sedimentation rate in a centrifugation field (Lustig et al., 2000). A more careful molecular mass determination is the mass spectrometry measurement, reviewed in Lin et al. (2003). Thanks to its high

fidelity of results and the wide range of sequences available in databases, it is the preferred method for protein identification, where possible and necessary.

In mass spectrometry, the sample (e.g. a tryptic digest of a protein spot) is ionised and mass per charge is analysed. The most commonly used ionisation techniques for polypeptides and peptides are electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI). In ESI, polypeptides are ionised amino acid by amino acid, and thereby sequenced according to mass per charge ( $m/z$ ). In MALDI, whole peptides are charged and their  $m/z$  is measured. More elaborate techniques include ion trap mass spectrometry (ionising a complex mixture of proteins, trapping them according to  $m/z$  and sending them one by one for further analysis in a tandem coupled mass spectrometer). To decrease the level of complexity at analysis, a pre-separatory technique such as HPLC is often used when peptide mixtures are studied. Presence of peptide fragments matching known sequences from databases will identify the protein. Two problems in database matching are that some different amino acids have the same mass per charge ratio, thus making them indistinguishable from one another, and that reported sequences in the databases may contain a substantial amount of erroneously annotated entries, thus making proteins with incorrect reference data evade identification. Sequencing errors may also make the databases references incorrect. Mass spectrometry is gaining further in popularity due to lowered costs of analysis. It is not limited to protein identification, but in proteomics it has had one of its greatest impacts as it offers new accurate possibilities of identification.

The immunological approach of identification is based on the production of specific antibodies and detection by a secondary antibody linked to an enzyme. The approach relies on the specificity of the antibody used; it is often produced against a short polypeptide sequence taken from the protein. If the polypeptide against which the antibody was raised was a long polypeptide sequence, there may be a population of antibodies so that many other proteins with sufficiently similar epitopes cause unspecific binding. If a sample includes several isoforms from a family of proteins with a high degree of similarity, the different isoforms may not be distinguished immunologically.

### **Identification and validation of subproteomes**

Whole cell membrane fractions may be identified by their protein composition. The membrane fraction is then identified from components that are known to be typical and unique for that particular membrane type. For example, in this thesis, membrane fractions enriched in plasma membranes are analysed. When a membrane subproteome is to be analysed, the membrane first has to be enriched. Purity assessment is difficult if compartment markers can be found in more than one compartment.

The plasma membrane fraction is often more easily distinguished from the chloroplast membrane fraction and the mitochondrial membrane fraction than from the tonoplast fraction. This is due to the presence of unique marker components in the chloroplast fraction (chlorophyll and other distinguishable pigments) and the mitochondrial fraction (cytochrome c oxidase is unique for mitochondria), but tonoplast markers sometimes can be present in plasma membranes and other membrane structures, which will be discussed later.

A soluble chloroplast protein, ribulose biphosphate carboxylase/oxygenase – Rubisco – presents an example of a problem in assessing the purity of a membrane fraction. Rubisco is a soluble protein complex that functions in the Calvin cycle, and is believed to be unique to the chloroplasts. Nevertheless, it has been shown to associate to membrane components, though probably in an unspecific manner. In a study where integral chloroplast membrane proteins were purified from soluble chloroplast proteins, Rubisco was co-purified with the hydrophobic proteins, even though it is not known to have any association with membrane components *in vivo* (Seigneurin-Berny et al., 1999). This is an example that illustrates that the presence of a putative soluble marker component may not always correlate with the general presence of soluble components. Integral marker components such as membrane proteins or pigments may be more reliable than soluble markers.

It should always be remembered that the more sensitive the method of analysis, the greater is also the risk that low abundance components that are contaminants from another fraction are interpreted as components of the fraction intended to be studied (van Wijk, 2001).

### **General considerations on membrane protein solubilisation**

Hydrophobic proteins and hydrophobic protein complexes are embedded in biological membranes. To study the proteins, they first have to be solubilised. For solubilisation, detergents – characterised by their ability to display one hydrophilic part and one hydrophobic part, and being able to form micelles – are used. Detergents can be crudely divided into ionic, zwitterionic (amphoteric) and non-ionic detergents (Welling et al., 1990).

Different detergents have different use and no detergent appears to be the optimal choice for all proteins of a certain category of proteins (Jones, 1999; Garavito & Ferguson-Miller, 2001). The characteristic properties of the detergent may be influenced not only by the hydrophobic and hydrophilic parts of the detergent, but also by detergent micellar shape, pH (Otzen, 2002), ionic strength, osmotic environment (Walter et al., 2000), and temperature (Sivars & Tjerneld, 2000). Also, the biological membrane thickness (McIntosh et al., 2003) and the lipids with which the detergent is interacting influences its solubilising properties (López et al., 2002). As a rule, ionic detergents, such as SDS, are more effective in solubilising proteins, but milder, non-ionic detergents, such as Triton X-100, are often required to preserve biological activity (Welling et al., 1990). However, in the broad groups of ionic, zwitterionic and non-ionic detergents respectively, there may be great differences between protein solubilisation efficiency and capacity for preserving quaternary structure and biological activity. Therefore, when different detergents such as dodecyl maltoside (Schägger & von Jagow, 1991), Triton X-100 (Poetsch et al., 2000) and digitonin (Giraud et al., 2002), each considered good and mild solubilisers, are tested, different patterns of proteins or protein complexes may be revealed, both qualitatively and quantitatively (Eubel et al., 2003; Grandier-Vazeille & Guérin M., 1996). Another aspect is that a detergent that is suitable for solubilising a particular component for analysis by one methodology may be less suitable when the same component is to be solubilised with another methodology (Poetsch et al., 1999). Also, additives such as glycerol may have an

effect upon the solubility of the detergent and hence its effective concentration (Eriks et al., 2003).

SDS is a highly efficient but denaturing solubiliser and is a standard detergent for denaturing PAGE. When it has been used for solubilisation, it is normally difficult to remove the detergent and restore enzymatic activity. For  $\text{Ca}^{2+}$ -ATPase from rat plasma membranes, however, it was possible to exchange the denaturing detergent for the milder detergent dodecyl maltoside, thus showing that it is possible to use properties of several solubilisers in a series (Dong et al., 1997). Shifts in equilibrium of denaturation state due to the SDS mole fraction in mixed SDS/dodecyl maltoside micelles could be monitored for the disulfide bond reducing protein, a bacterial membrane protein (Otzen, 2003). However, the applicability of detergent exchange leading to renaturation and restoration of activity may be substantially more limited for multicomponent protein complexes than for monomeric proteins. Therefore, for studies of native protein complexes, it may be a better option to employ only detergents that do not affect catalytic activity. This is a property that for instance dodecyl maltoside has when solubilising the abundant plasma membrane proton pumps (Johansson et al., 1994).

Even at maximum solubilisation that can be reached with a particular detergent (after sufficient incubation time), not all membrane proteins are solubilised. Membrane patches, called detergent-resistant membrane domains, exist and are hypothesised to constitute domain patches in the native membrane, establishing a lateral organisation in the membrane (Simons & Ikonen, 1997). Depending on cell type and detergent, plasma membranes may give rise to detergent-resistant membrane domains of different composition (Schuck et al., 2003). Subsets of membrane proteins, such as glycosylphosphatidylinositol(GPI)-anchored proteins, and the membrane lipids cholesterol and sphingolipids are overrepresented in these detergent-resistant membranes (Simons & Ikonen, 1997), indicating that they derive from distinguishable membrane domains. So, it is important to be aware of that a total and ultimate proteome of a particular membrane type is presently not possible to resolve, at least using mild detergents, which are required for several methods of analysis when examining membrane protein complexes. Interesting protein complexes may simply be inaccessible with the methods employed today.

Other components that influence the solubilisation of membrane bound proteins are chaotropic and kosmotropic compounds, which break the water structure (chaotropes) or order it (kosmotropes) and therefore influence the stability of the lipid phases of membranes (Koynova et al., 1997). In membrane protein solubilisation, the membrane destabilisation effect combined with high ionic strength by high concentrations of chaotropes such as urea or amino caproic acid (O'Farrell, 1975; Schägger & von Jagow, 1991) may be used to enhance the solubilisation of membrane proteins. In Appendix, the chaotropic/kosmotropic effect of NaCl, KI and urea upon the integrity of plasma membrane fraction protein complexes are discussed.

# THE PLANT PLASMA MEMBRANE

## **The plasma membrane is purified using aqueous two-phase partitioning**

The plasma membrane is the outermost membrane of the cell. It is the membrane type at which the work in this thesis has been aimed. Estimates indicate that approximately 3 % of the total proteins from plants may be plasma membrane proteins, assuming that plant protein distribution is similar to that of other organisms (Masson & Rossignol, 1995). This was also indicated from the maximum plasma membrane purification ratio as judged from abundant plasma membrane proteins (Sussman, 1994).

The most widely used method for purification of plasma membranes is by aqueous two-phase partitioning, described in Larsson et al. (1987). The method uses the distribution of components in a dextran/polyethylene glycol system, influenced by the salt content in the system. Polymer two-phase partitioning gives a partitioning among membranes of different origin, having right-side-out (i.e., cytoplasmic side-in) plasma membrane vesicles partitioned mainly to one (the polyethylene glycol enriched) of the phases.

## **Plasma membrane proteins and protein complexes**

All cell import and export of chemical compounds must involve the plasma membrane. Many physical stimuli from the environment, such as chemical compounds or a tension to be sensed, must also be perceived in the plasma membrane and transmitted to the inside of the plasma membrane to give an ordered response. Likewise, any signal whose response requires changes in cell structure, such as directed growth in auxin response or the formation of a cell plate upon cell division, demands that the membrane components are to some extent organised in a non-random structure. Many of the signal transducing components would be expected to be found at a relatively low copy number, whereas structural components are normally abundant.

The most abundant proteins in plasma membrane preparations are the plasma membrane intrinsic proteins (PIPs), also known as aquaporins (Johansson et al., 2001). These generally transport water, but some members of the aquaporin protein family may have other functions, e.g. urea channels (Liu et al., 2003) or glycerol channels (Biela et al., 1999). Even a blue light receptor function through flavin binding has been proposed (Lorenz et al., 2003). Aquaporins are also found in the tonoplast and are there termed tonoplast intrinsic proteins (TIPs). The whole family of aquaporins is called major intrinsic proteins (MIPs) and has 35 members in *A. thaliana* (Johansson et al., 2001).

Another highly abundant plasma membrane protein is the H<sup>+</sup>-translocating ATPase, a protein of approximately 100 kDa (Sussman, 1994; Palmgren, 2001; Morandini et al., 2002) that takes part in ion transport, pH regulation and growth. It is one of the most thoroughly investigated plant plasma membrane proteins. The plasma membrane H<sup>+</sup>-ATPases are encoded by a gene family with 12 members in *A. thaliana* named *aba1* through *aba12* and no cell investigated has been found to completely lack gene products from the gene family (Palmgren, 2001).

The plant plasma membrane contains several characterised proteins that have been suggested as candidates for protein complexes of high abundance. Cellulose synthase ( $\beta$ -(1-4) glucan synthase) is a complex that shows six-fold symmetry, indicating that it should be oligomeric. The catalytic subunit CesaA has a predicted molecular mass of 110 kDa but has in denaturing studies migrated as a 130 kDa protein (Kimura et al., 1999). Other components purified with cellulose synthase activity in different studies have included polypeptides of 28-35 kDa, 37 kDa, 55 kDa, 52-57 kDa and 70 kDa. Evidence has been found for non-catalytic subunits in the synthase complex. A working model for cellulose synthase action includes tight interaction with sucrose synthase (Delmer & Amor, 1995).

Callose synthase, also called glucan synthase or  $\beta$ -(1-3) glucan synthase, has previously been analysed by product entrapment after sucrose gradient centrifugation (Turner et al., 1998). Polypeptides co-sedimenting with callose synthase activity have been suggested as candidate partners in a callose synthase complex (Hong et al., 2001a; Turner et al., 1998). From physiological reasoning, sucrose synthase has been suggested a putative complex partner for both callose synthase and cellulose synthase (Amor et al., 1995). A callose synthase complex was shown to increase in size upon cation interaction and the complex had an increased activity when incubated with chelators of divalent cations. Both effects may be due to the association/dissociation of another protein to the complex, possibly the  $\text{Ca}^{2+}$ -binding protein annexin (Andrawis et al., 1993). However, there has been a lack of methods to conclusively determine what polypeptides are part of the callose synthase complex.

There are proteins first detected as cytosolic, for which at least a population has later been shown to be bound to the plasma membrane. An example is the above-mentioned sucrose synthase (Amor et al., 1995; Carlson & Chourey, 1996) which in plants occurs in a tetramer (Porchia et al., 1999).

### **The study of plasma membrane proteomes has been impeded due to the lack of suitable methods**

An approach for proteome analysis of plasma membranes that has been employed is IEF/SDS-PAGE on different cellular fractions (Santoni et al., 1999b). IEF/SDS-PAGE resolved proteins from purified plasma membranes were compared to soluble fraction proteins, mainly cytoplasmic, from the same source. Proteins that were enriched in the plasma membrane fraction were considered putative plasma membrane proteins. The approach is hence a kind of subtraction method for proteome studies. Different additives in the solubilisation step have been used for solubilisation of hydrophobic proteins (Santoni et al., 2000; Fountoulakis & Takács, 2001). Still, the approach did not solve the problem that many hydrophobic proteins are very difficult to solubilise using the detergents and chaotropes available and required for IEF/SDS-PAGE (van Wijk, 2001). Generally, membrane proteins are so difficult to solubilise and resolve on two-dimensional gels, that one-dimensional SDS-PAGE has been suggested as a better option for hydrophobic proteomic studies (Galeva & Alterman, 2002). The urea concentrations sometimes employed in IEF/SDS-PAGE (O'Farrell, 1975; Fountoulakis & Takács, 2001; Santoni et al., 2003; Krijgsveld et al., 2003) will normally denature proteins (Schoeffler et al., 2003), allowing resolution of single polypeptides but not of complexes.

## BN-PAGE ANALYSIS OF PLANT PLASMA MEMBRANES

### **BN-PAGE is a method to resolve membrane protein complexes**

BN-PAGE is an electrophoretic method for resolution by native size designed for use with mild detergents when studying hydrophobic proteins and membrane protein complexes. In the method, membranes are first resuspended in an amino caproic acid buffer that helps keeping the protein complexes intact upon solubilisation and electrophoresis. Protein complexes are solubilised using a mild detergent such as dodecyl maltoside (Schägger & von Jagow, 1991), Triton X-100 (Poetsch et al., 2000) or digitonin (Schägger & Pfeiffer, 2000). After pelleting the unsolubilised material, the dye Coomassie Brilliant Blue G-250 is added, exchanging for the initial detergent. The Coomassie introduces a negative charge-shift that enhances the migration of the proteins in a gradient native gel system (Schägger & von Jagow, 1991). After native gel electrophoresis, the protein complexes are denatured and solubilised using SDS and a reducing agent. The subunits can then be resolved according to denatured molecular mass by a second dimension SDS-PAGE. This second dimension labels the method BN/SDS-PAGE. Consequently, BN-PAGE refers to the native dimension of the method and BN/SDS-PAGE refers to the denaturing dimension. Together, the two dimensions give information on the functional membrane protein complexes. A comparison between the methods using mitochondrial proteomes furthermore shows that BN/SDS-PAGE is significantly better at solubilising and resolving hydrophobic proteins as compared to IEF/SDS-PAGE (Heazlewood et al., 2003).

Proteins can be stained or analysed by western blotting after native dimension of BN-PAGE (Culvenor et al., 2004) as well as after the denaturing dimension (Rasmusson & Agius, 2001). Trypsin digestion followed by mass spectrometry, and other analysis methodologies often employed for IEF/SDS-PAGE and SDS-PAGE are also compatible with BN/SDS-PAGE.

Originally developed for mitochondrial membranes from animals (Schägger & von Jagow, 1991), BN/SDS-PAGE was shown to work equally well for plant mitochondria (Jänsch et al., 1996) and has been used for proteome studies on chloroplast membranes (Kügler et al., 1997), thus extending its utility and establishing that the technique can be valid for several membrane types. It has also been used to study algal thylakoid protein complexes (Rexroth et al., 2003) and to resolve prokaryotic membrane protein complexes (Heuberger et al., 2002). Using BN/SDS-PAGE, a great number of respiratory complex components have been resolved and confirmed through identification (Devreese et al., 2002). The charge-shift basis of the method has been employed for IEF-PAGE as well (Hird et al., 2000) but the results are not of the same resolution as those for BN/SDS-PAGE. The BN/SDS-PAGE technique has furthermore been adopted for minigel systems, thus increasing the throughput of the analysis of mitochondrial membrane protein complexes (Rasmusson & Agius, 2001; Brookes et al., 2002).

BN-PAGE preserves the biochemical activity of the components separated after electrophoresis (Schägger et al., 1994). Direct activity studies include in-gel staining for

NADH-reductase activity (Cardol et al., 2002). BN-PAGE is preferably used for studying protein complexes, as in its first dimension separation is based on native molecular mass, where the gel composition is often chosen to allow separation at high molecular masses rather than the mass ranges more typical for small monomeric proteins. Thus, it does not aim at giving a good resolution for monomers or components of low native molecular mass, as such components would necessarily convert the technique into basically a one-dimensional SDS-PAGE with an introduced extra step where possible artifacts may occur. The method allows separation and resolution of hydrophobic complexes, e.g. mitochondrial complexes I-V, thus making integral membrane protein complexes the proteins of choice to study using BN/SDS-PAGE.

Combined use of BN/SDS-PAGE with antibody immunodetection has been useful for revealing that subunits (NdhI, NdhJ and NdhK) with sequence similarities to the subunits of NADH:ubiquinone oxidoreductase (the so called mitochondrial Complex I) are present in a thylakoid membrane complex with a high native molecular mass (Burrows, 1998). BN/SDS-PAGE combined with mass spectrometry, was used to establish the composition of the preprotein translocation pore complex of the outer mitochondrial membrane in *A. thaliana*, both with respect to subunit content and with respect to native molecular mass of the complex (Werhahn et al., 2003). BN/SDS-PAGE has also been refined as a native dimension from which the subunits from a resolved complex were electroeluted and subjected to SDS-PAGE resolution. Conclusions could be drawn with respect to band pattern of the chloroplast F<sub>0</sub>F<sub>1</sub>-ATPase (Neff & Dencher, 1999). However, this approach is perhaps not to be recommended without combination to some other principle in the analysis such as mass spectrometry or western blotting, as BN/SDS-PAGE is a separation method where the total proteome is of great value in the result verification and validation. The preparative approach described above is though a useful way to employ BN/SDS-PAGE.

Using BN/SDS-PAGE on mitochondria, bands of higher native molecular mass than expected were found. An approach for testing whether the native BN-PAGE electrophoresis itself generally causes artifactual aggregates of protein complexes was done by cutting out first dimension protein complex bands corresponding to these putative supercomplexes – i.e. complexes with functional channelling between them – of the mitochondrial F<sub>0</sub>F<sub>1</sub>-ATPase, then subjecting them to a second dimension denaturing electrophoresis and comparing the results of each putative supercomplex (Arnold et al., 1998). This supercomplex had additional components; a strong indication that it was not a mere artefactual aggregation. Resolution of supercomplexes using one mild detergent in a first native dimension followed by the resolution of this supercomplex in a second native dimension using another mild detergent allowed the detection of several supercomplexes with distinct stoichiometries (Schägger & Pfeiffer, 2000). However, some care should be taken before interpreting the validity of inactivated forms of complexes, as an investigation on the dimerisation and inactivation of bovine heart mitochondrial F<sub>0</sub>F<sub>1</sub>-ATPase using Triton X-100 as a detergent (Tomasetig et al., 2002) does not correspond to a comparable inactivated state of the oligomers after digitonin extraction of yeast F<sub>0</sub>F<sub>1</sub>-ATPase (Paumard et al., 2002). Hence, it is possible that the detergent itself may influence the behaviour of a complex, or that the same complex behaves differently in different organisms, such as is described for the H<sup>+</sup>-ATPase interaction with 14-3-3 protein and fusicoccin after transformation in yeast (Piotrowski et al., 1998).

An interesting combination of techniques was presented for mitochondrial membrane proteins: a first dimension of BN-PAGE with individual protein complex bands cut out and subjected to IEF/SDS-PAGE so that a three-dimensional approach was applied (Werhahn & Braun, 2002). This method should, at least theoretically, avoid the shortcomings of solubilising hydrophobic proteins that IEF/SDS-PAGE itself suffers from (van Wijk, 2001) at the same time as it is providing increased resolution of components contributing to the protein complexes. However, the study of Werhahn & Braun (2002) also shows that IEF/SDS-PAGE will not resolve a particular component as one protein spot, but rather as a series of spots with a more or less wide pI distribution as shown in O'Farrell (1975) and Santoni et al. (2003). It may be difficult to tell whether several different isoenzymes and posttranslationally modified proteins can be present in a complex or if this result indicates that IEF/SDS-PAGE causes artefactual spots.

For mitochondria, general membrane proteome patterns from BN/SDS-PAGE analyses have been established. The prominent protein complexes appear with a predictability that allows using them as references for native molecular mass (Jänsch et al., 1996; Ludwig et al., 1998; Schägger & Pfeiffer, 2000; Brookes et al., 2002; Nijtmans et al., 2002; Lin et al., 2002; Heazlewood et al., 2003).

### **The solubilisation of plant plasma membranes on BN/SDS-PAGE**

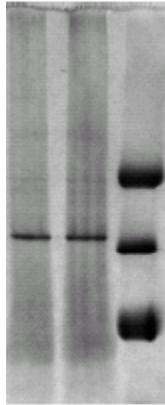
In order to investigate whether BN/SDS-PAGE could be used for resolution of the abundant proteins in the plant plasma membrane proteome, membrane fractions enriched in plasma membranes (Larsson et al., 1987) were solubilised under different conditions and subjected to BN/SDS-PAGE.

Fig 1 shows that for several proteins, an improved solubilisation of protein complexes from plasma membrane fractions is obtained using twice the concentration of aminocaproic acid buffer compared to published mitochondrial solubilisations (Schägger et al., 1994; Jänsch et al., 1996; Kügler et al., 1997; Grandier-Vazeille & Guérin, 1996; Heuberger 2002). Resolution is improved upon higher ionic concentration, with components not seen at lower ionic strength. The quantitative difference is best seen when Fig 1 B and Fig 1 C are compared.

Using BN-PAGE, the efficiencies of two non-ionic detergents,  $\beta$ -D-dodecyl maltoside and Triton X-100, were compared (Fig 2, 3). Different concentrations of the detergents were used to determine optimal concentration. In the study, an identical amount of membrane as judged from protein content measurements (Bearden, 1978) was used for all detergents and concentrations tested.

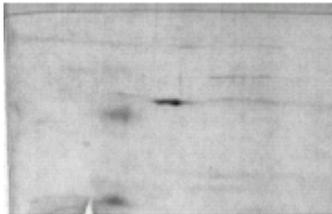
## Fig 1: The effect of increasing ACAB concentration

A 1 2 3



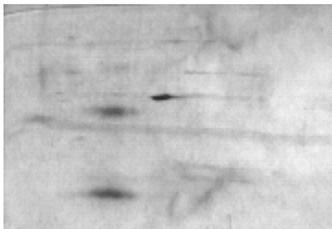
**Figure 1A:** The figure depicts PM isolated from *Spinacia oleracea*. PM was solubilised in either ACAB (Schägger & von Jagow, 1991) or 2xACAB for native resolution (BN-PAGE). 2 % (w/v) dodecyl maltoside was used for solubilisation. Lane 1 is with ACAB (750 mM aminocaproic acid, 50 mM BisTris, pH 7.0), lane 2 is with 2xACAB (1.5 M aminocaproic acid, 100 mM BisTris, pH 7.0). Identical amounts of protein was used as starting material. Lane 3 is molecular mass standard (thyroglobulin 669 kDa, ferritin 440 kDa, catalase 230 kDa).

B



**B:** Second dimension resolution (BN/SDS-PAGE) of the 1xACAB solubilised plasma membranes. Lane 1 from Fig 1A was used for second dimension in Fig 1B. Increasing native molecular mass is from left to right.

C



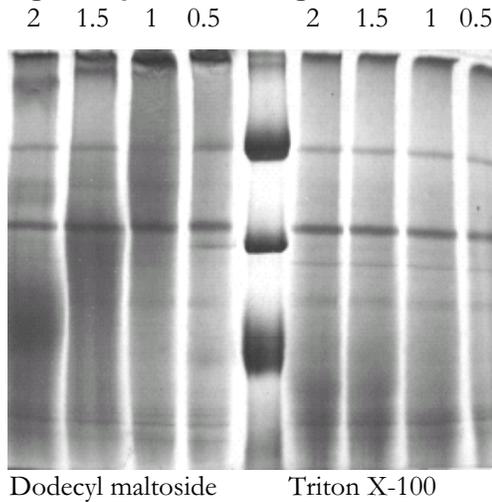
**C:** Second dimension resolution (BN/SDS-PAGE) of the 2xACAB solubilised plasma membranes. Lane 2 from Fig 1A was used for second dimension in Fig 1B. Low native molecular mass is to the left, high native mass is to the right. A comparison with Fig 1B shows that there is more material resolved when 2xACAB is used.

$\beta$ -D-dodecyl maltoside was found to be a more efficient solubilising agent for abundant hydrophobic plasma membrane protein complexes compared to Triton X-100 (Fig 2). The bands between 670 kDa and 440 kDa, for example, are prominent when dodecyl maltoside is used but invisible upon Triton X-100 solubilisation. Also, the component giving the diffuse but prominent band at 230 kDa is considerably better solubilised with dodecyl maltoside than with Triton X-100.

The detergent concentration dependence is not unexpected; for dodecyl maltoside it has been shown to be almost linear with respect to both detergent concentration and amount of lipid material to solubilise (López et al., 2003). The different solubilising capacity of lipids for different detergents distinguishes the results when two different detergents are

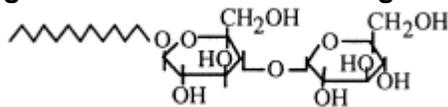
used in an experiment (Schuck et al., 2003). Therefore, the appearance of a band near 400 kDa that is better resolved after Triton X-100 solubilisation than after dodecyl maltoside solubilisation (Fig 2) is likely to be an example of that no detergent is a better solubiliser for all proteins in a proteome. It is possible that there are solubilised complexes in the dodecyl maltoside lanes that are covered by another solubilised, more abundant, band in that area, but that is speculative.

**Fig 2: Impact of detergent choice and concentration**

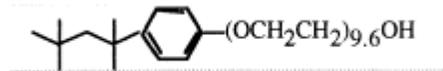


**Figure 2:** Different concentrations (w/v) of detergents were used to solubilise plasma membrane protein complexes from *A. thaliana* in two series on a native (4-14.5 %) gel:  $\beta$ -D-dodecylmaltoside (2 %, 1.5 %, 1 %, 0.5 %) and Triton X-100 (2 %, 1.5 %, 1 %, 0.5 %). Standard proteins (thyroglobulin 670 kDa, ferritin 440 kDa, catalase 230 kDa, lactate dehydrogenase 140 kDa) were loaded in the central lane. The gel shows that dodecyl maltoside is generally a better solubilising agent than Triton X-100 for BN/SDS-PAGE on plasma membranes.

**Fig 3: Structure of used detergents**



$\beta$ -D-dodecyl maltoside



Triton X-100

Detergents are not expected to be able to solubilise all material. It has been shown that  $\beta$ -D-dodecyl maltoside solubilises approximately 60-70 % of total plasma membrane fraction proteins, but has the capacity to solubilise some proteins, such as the plasma membrane  $H^+$ -ATPase, better than this (Johansson et al., 1994), and hence solubilises some proteins at a lower yield. Hence, it is possible that some protein complexes are overrepresented in the gels.

Different mild detergents may solubilise protein complexes in a qualitatively different way due to their different characteristics. This may give rise to different apparent molecular masses (Grandier-Vazeille & Guérin, 1996). In IEF-SDS/PAGE, great differences in protein pattern is also evident for different (denaturing) zwitterionic and non-ionic detergents (Luche et al., 2003). Hence, the effect of different detergents giving rise to different apparent molecular masses appear for mild detergents, but is not confined to mild detergents. This possible migration change of complexes calls for a denaturing dimension to show whether a protein complex is missing after solubilisation with a

detergent, or if it may appear as a complex of slightly different native molecular mass. However, such migratory changes should be minor.

The resolution difference at different detergent concentrations seen in Fig 2 shows that the detergent concentration is important for solubilisation, as is the ionic strength (Schägger & von Jagow, 1991; Fig 1, this thesis). Some studies conducted by others have used a high detergent concentration (Manting et al., 2000), whereas several other studies have employed detergent concentrations corresponding to the lower concentrations used in Fig 2. Even lower detergent concentrations than that have also been used to solubilise protein complexes in either a low ionic strength buffer (Culvenor et al., 2004; Bacher et al., 2002; Zolla et al., 2002; Sazanov et al., 1998; Ghaim et al., 1997; Turner et al., 1998) or in a high ionic strength buffer (Cardol et al., 2002) or both lower concentrations and the concentrations used in Fig 2 with a low ionic strength (Heuberger et al., 2002). However, for proteome studies, high ionic strength and high detergent concentration appears to be the best combination.

Near several of the standard molecular masses, sharp complex bands were found in the lanes with the samples (Fig 2). Bands are visible at 670 kDa and 440 kDa. However, these should not be contaminants from the standard proteins, as that would require a general contamination; that bands appear near all the standard complexes, in proportion to the standard proteins. This is not the case, as an easily recognisable band would then appear at 230 kDa, in visibility comparable to the one at 670 kDa.

It appears as if diffuse protein complexes are shifted to lower apparent native molecular mass when higher dodecyl maltoside concentrations are used (Fig 2). This could be due to incomplete solubilisation with mixed micelles containing different amounts of lipids and possibly more than one complex.

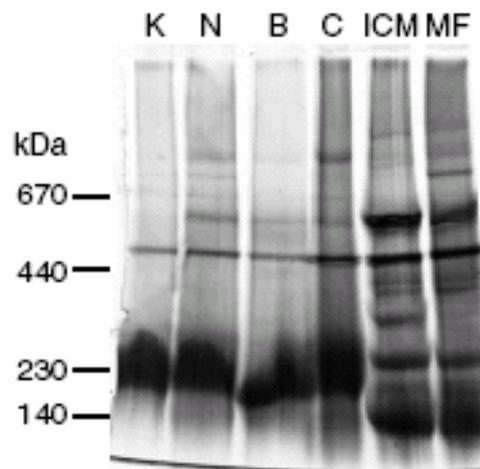
### **BN-PAGE resolves multiple high molecular weight protein complexes in plasma membrane fractions**

A set of BN/SDS-PAGE (Schägger & von Jagow, 1991) experiments was carried out on leaf plasma membranes from spinach (*Spinacia oleracea*). This is described in Appendix. BN/SDS-PAGE has not been used for plasma membrane proteome studies previously. In the study, it was also examined to what extent different membrane treatments could wash off peripheral subunits and proteins loosely attached to the membrane from the complexes (Fig 4 and Fig 5).

Salts of different kinds can have effects on membranes and protein complexes. In general, ions will destabilise electrostatic interactions between proteins and membranes. Chaotropic ions such as I<sup>-</sup> destabilise protein complexes (Hatefi & Hanstein, 1974). However, Na<sup>+</sup> ions may instead stabilise membranes (Böckmann et al., 2003). Other chemical compounds, such as urea, may at high concentrations cause denaturation of proteins (Schoeffler et al., 2003) and consequently alter protein complexes significantly. Therefore, washing membrane fractions with salts that have different properties could give information on the nature of the strength of interactions between the proteins in each complex and between the protein complex and the membrane.

Plasma membrane fractions from *Spinacia oleracea* were prepared essentially as described in Larsson et al. (1987) using a 6.1 % (PEG, dextran) two-phase aqueous partitioning system and were stored at -80°C prior to use for BN/SDS-PAGE. Plasma membranes were treated and pelleted with Brij 58 (B) or without Brij 58 (C). Plasma membranes were also treated and pelleted in the presence of Brij 58 and NaCl (N) or Brij 58 and KI (K). Brij 58-washing turns plasma membrane vesicles inside-out (Johansson et al., 1995). For optimum solubilisation and electrophoresis of the plasma membrane protein complexes (as shown in Fig 1 and 2), 2 % dodecylmaltoside and 2xACAB was used upon resuspension of the pellets. To study differences in proteome pattern, intracellular membrane fractions (Larsson et al., 1987) and microsomal membranes were studied with the same system for comparison.

**Fig 4: Resolution in native dimension**



**Figure 4:** The figure shows native dimension resolution of protein complexes after different treatments. An equal amount of *Spinacia oleracea* plasma membrane was used as a starting material before treatment for K (Brij 58, 0.5 M KI), N (Brij 58, 1.0 M NaCl), B (Brij 58) and C (control). Brij 58 was used at a protein:detergent ratio of 1:10). ICM (intracellular membranes) and MF (microsomal fraction) was also loaded onto the native gel for separation of protein complexes. Gels were Coomassie stained. Native standards are as in Fig 2 and native molecular masses are indicated to the left.

As is seen from Fig 4, the intracellular membrane fractions and microsomal fractions show a similar pattern of protein complexes, different from the plasma membrane fractions. However, the complexes present in plasma membrane fractions also faintly appear in intracellular membrane fractions and microsomal fractions. This is not unexpected (Widell & Larsson, 1990). The expected pattern is that the abundance of plasma membrane proteins decreases in intracellular membranes compared to microsomal membranes. The results are thus consistent with that the plasma membrane has a unique set of protein complexes. It is from Fig 4 evident that membrane washing with NaCl and Brij 58 prior to BN/SDS-PAGE appears to sharpen the protein complex bands and have increasing resolution on the gel. On the other hand, in KI/Brij 58 washed membranes, several bands have disappeared, indicating severe destabilisation of the protein complexes.

Throughout all plasma membrane fractions, it is evident that a highly abundant protein complex near 200 kDa apparent molecular mass is not washed off, implying that it is a very stable integral membrane protein complex. A protein complex located near 500 kDa apparent molecular mass is strong throughout the gel, including the non-plasma-membrane-enriched fractions. This was later identified as Rubisco, which here apparently remains in all fractions.

The difference in release of total membrane proteins between treatment with NaCl and KI is not unexpected, as I<sup>-</sup> is known to have strong chaotropic characteristics, whereas neither Na<sup>+</sup> nor Cl<sup>-</sup> has such characteristics (Hincha, 1998). 0.5 M KI has in other studies been sufficient for dissociating peripheral components from membranes (Arai et al., 1988). NaCl has also been shown, as opposite to chaotropes such as urea, to stabilise mixed micelle structures (Walter et al., 2000), which should help keeping the integrity of membrane protein complexes during the Brij 58 wash.

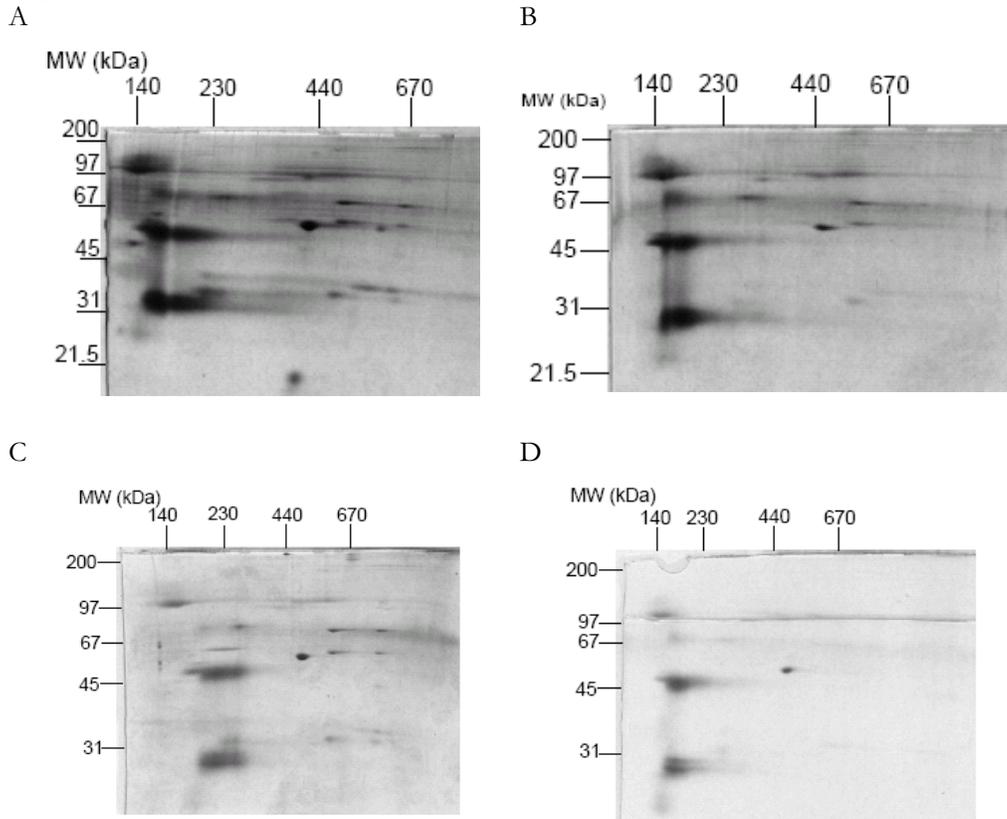
Though a general loss in quantity of components is seen upon Brij 58 wash (Fig 5), no band is completely removed. This implies that the plasma membrane fraction abundant protein complexes are all plasma membrane components and not components enclosed in the vesicles.

As a washing alternative to NaCl and KI, 4 M urea (data not shown) was also tested using the same protocol. Known to be a strong chaotrope with protein denaturing properties when applied at high concentrations (Schoeffler et al., 2003), not many protein complexes were expected to remain after the urea washing. The only complex that remained abundant after urea wash was the 200 kDa abundant native protein complex.

Upon denaturing resolution (Fig 5) of the BN-PAGE lanes in Fig 4, the most prominent proteins of PM enriched fractions were found near a native mass of 100-200 kDa. Identified components from Fig 5 C are shown in Fig 6 and the identification through mass spectrometry is more thoroughly presented in Appendix. At 150 kDa apparent molecular mass, a monomeric form of H<sup>+</sup>-ATPase was found (A100). This was expected, since it is known as one of the major protein constituents of the plasma membrane. An oligomeric form of the H<sup>+</sup>-ATPase, was identified at an apparent native molecular mass of approximately 600 kDa (F100). At an apparent native molecular mass of approximately 200 kDa, the most abundant plasma membrane protein complex was found. It was resolved as three contributing components of approximately 30 kDa, 55 kDa and 85 kDa denatured molecular mass, the highest mass form of considerably lower abundance than the other two. However, upon western blotting of the denaturing dimension of the PM control gel, all three components were immunologically reacting with antibodies raised against the major intrinsic protein PM28A (aquaporin). This implies that it is the monomer (28 kDa), a dimer (56 kDa) and a trimer (84 kDa) of the same complex subunit that appears due to incomplete denaturation in the second dimension. At an apparent native molecular mass of 320 kDa, a protein identified as an ERD4-like protein was found (D81). V-ATPase subunits E (I 32), C (I 44) and B (I 65) were identified in one complex at a native molecular mass of 800 kDa. At a slightly lower molecular mass, a protein with sequence similarity to HIR (hypersensitivity induced reaction) proteins was identified as spot K35. Glucan synthase was also sequenced (H190) near 1000 kDa apparent native molecular mass. An additional putative subunit of a glucan synthase complex having the

same native migration was found at approximately 130 kDa.

**Fig 5: The effect of washing with salt**

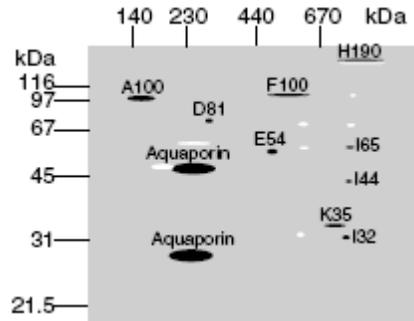


**Figure 5:** Two-dimensional (BN/SDS-PAGE) resolution of plasma membranes. The lanes correspond to those in Fig 4. A is control plasma membranes, B is Brij 58 washed membranes, C is plasma membranes washed with Brij 58 and 1 M NaCl, D is plasma membranes washed with Brij 58 and 0.5 M KI. All gels were stained using Coomassie. For protein identifications, see Fig 6.

A catalytic subunit of glucan synthase complex, having 16 transmembrane helices (Hong et al., 2001b), has been identified and is expected to be highly hydrophobic. Heuberger et al. (2002) have suggested that a correction factor of 1.8 is to be introduced in BN-PAGE for highly hydrophobic proteins due to increased detergent interaction, leading to a higher apparent native molecular mass upon BN-PAGE. Using this correction factor, the complex should be interpreted as 550-600 kDa. There were at least one co-migrating subunit, of slightly lower molecular mass. However, this mass did not closely correspond to the previously reported callose synthase complex additional proteins of 110 kDa (Turner et al., 1998), a 57 kDa UDP-glucose transferase (Hong et al., 2001b) or phragmoplastin which migrates as a polypeptide of less than 90 kDa (Hong et al., 2001b). To speculate, a native molecular mass of 550-600 kDa obtained here after correction corresponds well to a complex consisting of two subunits of the 190 kDa and two

subunits of the lower molecular mass. The 1:1 stoichiometry is supported by the relative staining intensity between the two subunits. Hence, there is an indication that the glucan synthase complex found with BN/SDS-PAGE has a subunit of 190 kDa and a co-migrating subunit that may previously not have been reported for the glucan synthase complex.

**Fig 6: Identified polypeptides**



**Figure 6:** Protein bands corresponding to Fig 5 C were cut out and subjected to mass spectrometry (MS/MS). One of the components, the aquaporin, was identified using an antibody against the aquaporin PM28A. The data is also presented in Appendix.

The higher oligomeric form of H<sup>+</sup>-ATPase (F100) was confirmed also for *A. thaliana* (see below). The F100 has a native molecular mass four times as high as that of the monomeric form of H<sup>+</sup>-ATPase (A100), i.e., it appears to have a tetrameric arrangement. This 4:1 native mass ratio holds for all studies made in the work presented here. No other H<sup>+</sup>-ATPase complex candidate protein is seen with this reproducibility in the gels. Hence, BN/SDS-PAGE concludes a tetrameric oligomeric form of H<sup>+</sup>-ATPase for both *S. oleracea* and *A. thaliana*.

H<sup>+</sup>-ATPase has been reported as a protein that can catalytically function as a monomer (Goormaghtigh et al., 1986) but has in other species reconstituted as multimeric catalytically active forms (Briskin & Reynold-Niesman, 1988). The H<sup>+</sup>-ATPase is regulated through 14-3-3 protein (Oecking et al., 1997; Svennelid et al., 1999) and it is then possible for the fungal toxin fusicoccin to bind to the complex (Baunsgaard et al., 1998; Oecking & Hagemann, 1999). It has been reported that H<sup>+</sup>-ATPase itself has higher oligomeric forms, differing between dimer (Briskin & Reynold-Niesman, 1988), trimer (Anthon & Spanswick, 1986) and hexamer (Chadwick et al., 1987). When expressed in yeast, the plant H<sup>+</sup>-ATPase was detected at molecular masses indicating an oligomeric composition, as opposed to the yeast H<sup>+</sup>-ATPase in the same study (Piotrowski et al., 1998), indicating that H<sup>+</sup>-ATPase from different organisms may be differently organised *in vivo*.

### **Tonoplast proteins are frequently found in plasma membrane fractions**

It may at a first glance seem surprising to find V-ATPase (a tonoplast protein complex) in plasma membrane fractions, but this has also been found by other investigators in proteome studies of plasma membrane fractions. The V-ATPase comprises a very significant fraction of the total tonoplast proteins, estimations ranging between 6-35 %,

but it has also been suggested to be present in other membranes, such as the plasma membrane (Ratajczak, 2000). Also, in cells from mice, studies have shown that upon stimulation, V-ATPase subunit *a* may co-localise with lysosomes that are transported to and fused with the plasma membrane. The transport route of V-ATPase was co-localised with the acidification of the compartments with which the V-ATPase was transported, indicating that the presence of subunit *a* also was accompanied by the presence of the functional complex (Toyomura et al., 2003). Whether this is also occurring in plant cells is still speculative, but the possibility should not be ruled out, since V-ATPase subunits A, B, C, D, E and G have been found in plasma membranes in *A. thaliana* (Santoni et al., 2000) as well as peribacteroid membranes (*L. japonicus* infected with *Rhizobium*) have contained V-ATPase (Wienkoop & Saalbach, 2003).

The most abundant tonoplast proteins are often the tonoplast intrinsic proteins (TIPs) (Maeshima, 2001), which are members of the aquaporin protein family (Johansson et al., 2001). The TIPs are mainly localised to the tonoplast, but there is evidence for presence of TIPs in the plasma membrane as well as other internal membranes in *A. thaliana* (Liu et al., 2003).

In Fig 5, it is in all gels clearly observable that a component of approximately 23 kDa and another weak component of twice that mass, constituting a complex with a native molecular mass of 90 kDa, when hydrophobicity is compensated for by dividing apparent native mass by 1.8. The native complex molecular mass corresponds very well to a tetrameric structure of the 23 kDa component. The components are of much lower abundance than the aquaporin components, but always present, and a speculation is that they may, though not yet identified, represent monomeric and dimeric (incompletely solubilised) TIP. Localisation mainly in another membrane, possibly the tonoplast, of this complex is supported by the relatively much stronger presence in intracellular membrane fractions (data not shown). TIPs that have also been localised to the plasma membrane have been predominantly localised to the tonoplast (Barkla et al., 1999), just as is suggested here.

A study of intracellular membrane fractions and microsomal membrane fractions (see Appendix) reveals that most protein complexes appearing in these two gels are completely depleted from plasma membrane fractions. Furthermore, the protein complexes appearing in the plasma membrane fractions are with the exception for Rubisco of a lower relative abundance in the intracellular membrane fraction than in the microsomal fraction, indicating that, as expected, the enrichment effects are substantial.

## **BN-PAGE IN FUNCTIONAL COMPARISON**

### **Cold treatment effects on plasma membrane protein complexes visualised by BN/SDS-PAGE**

Plants under stress exhibit some common responses, whether it is cold stress, drought stress or salt stress (Browse & Xin, 2001; Thomashow 2001; Zhu 2001). The similarity in the other responses may be due to that drought stress and chilling stress often lead to an

osmotic stress situation, but it is more probable that the responses are themselves similar, as experiments using cold but non-freezing stress, which should not lead to dehydration, also initiates these common responses (Zhu, 2001). To understand the stress responses and get an overview that allows the interpretation of single component analyses, transcriptomic, proteomic and metabolomic approaches may be used. A comparison of stress induction (where the expression level was at least doubled upon the stress) of gene expression monitoring 1300 genes showed that drought induced 44 genes, whereas 19 genes were induced in the same material by cold (Seki et al., 2001), showing that it really is different pathways that coincide in the different stress responses. In the study, more than half of the inducible genes were not previously reported as stress inducible genes and frequently but not in all cases, the cold induced genes were also induced by the drought stress (Seki et al., 2001).

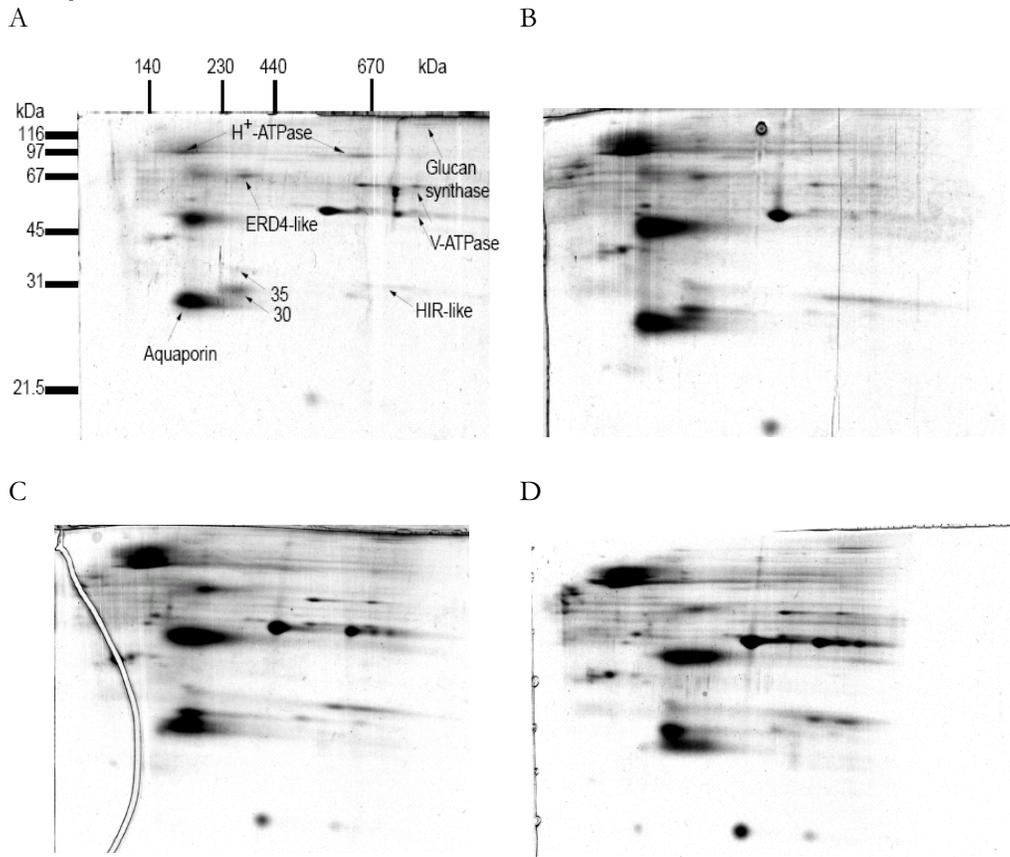
Cold acclimation, leading to an increased tolerance for freezing temperatures, of plants may occur when plants are subjected to low but non-freezing temperatures for days or weeks (Örvar et al., 2000; Browse & Xin, 2001), depending on the species studied. In studies, electrolyte leakage is used as a measure of the degree of freezing injury (Browse & Xin, 2001), so keeping the integrity of the membranes is one of the most important issues in cold acclimation. Therefore, it was hypothesised that cold treatment may affect the abundant protein complexes in the plasma membrane proteome.

For a physiological comparison of abundant plasma membrane fraction protein complexes, a cold treatment investigation was done. Spinach (*S. oleracea*) was grown in cold room (7°C, 10 hours of light per day) during the last days prior to harvest and leaf material was harvested using the plasma membrane purification protocol. A series of two-dimensional BN/SDS-PAGE was done with cold treatment for 7 days, 3 days and 1 day and an untreated control. The result is seen in Fig 7.

Cold treatment seemed to induce a shift in apparent native molecular mass of the aquaporin as compared to the migration of other abundant protein complexes (Fig 7). The shift is calculated to be approximately 40 kDa after mass correction for hydrophobicity (Fig 8). The reason for the shift requires more thorough study, using a resolution aimed at the area near 200-250 kDa apparent molecular mass. Possible causes are the addition of one component or several components to the aquaporin tetrameric structure or a change in expression of aquaporin genes, inducing gene products with a larger apparent native molecular mass. An alternative explanation is as follows.

Under chilling conditions, the level of unsaturation of membrane lipids is expected to increase (Taiz & Zeiger, 2002). As detergents solubilise by forming mixed micelles, it cannot be ruled out that a modified lipid composition after cold treatment may account for the apparent native molecular mass for the aquaporin due to tighter association with lipids after solubilisation. Higher detergent concentrations may be required to obtain the tendency of smaller mixed micelles, discussed above. However, if this is the case, then it indicates that the aquaporin may be located to membrane patches that differ in lipid composition from those where the other abundant protein complexes are found, as no other plasma membrane protein complex displays the change in apparent mass.

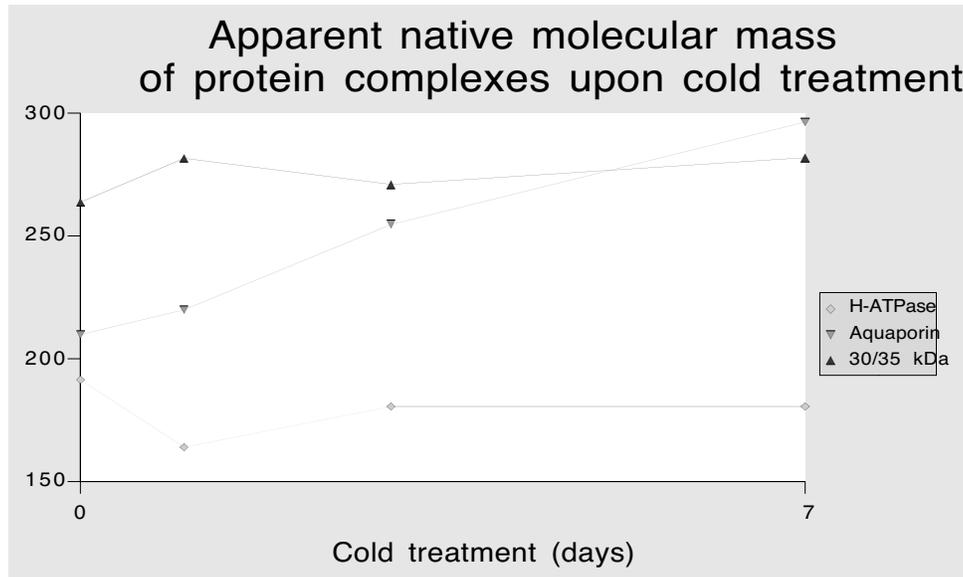
**Fig 7: The effect of cold treatment on plasma membrane protein complexes**



**Figure 7:** Plasma membrane fractions were harvested from spinach (*S. oleracea*) that were grown in a cold room (7°C) for the last day (B), the last 3 days (C) or the last 7 days (D) prior to harvest. (A) is a control plasma membrane fraction that was not subjected to low temperature. For all treatments, one representative gel out of two is shown. All gels, including replicates, used plasma membranes prepared separately. Native mass is shown horizontally, denatured mass is shown vertically.

A most notable difference that occurs at low temperature exposure is that a protein corresponding to the identified HIR-like protein (see Appendix), called K35 upon identification (Fig 6), increases greatly in abundance over the exposure time period (Fig 7). This implies a change that occurs by increasing gradually over time and is not transient like many other cold effects are (Plieth et al., 1999). Additionally, the protein corresponding to HIR-like protein has a denatured molecular mass similar to the larger subunit of a 30/35 kDa protein complex (requires a thorough study of Fig 7, with a correction for a skew in the protein pattern evident from streaking proteins) and it covaries with it in abundance. Therefore, it would be interesting to sequence both 30/35 kDa complex components to see if it may even be the same protein taking part of two different complexes.

**Fig 8: The aquaporin increases its native molecular mass after cold treatment**



**Figure 8:** Native molecular masses were measured and obtained from Fig 7. Time points are 0, 1, 3 and 7 days of cold treatment. Two relatively adjacent protein complexes were chosen as references to illustrate the shift in native molecular mass of the aquaporin complex after cold treatment (symbols indicated at the right y-axis; kDa of the native complexes on the left y-axis). Note that the aquaporin is the only complex that shifts molecular mass. The stability in native molecular mass is a characteristic of all protein complexes found in Fig 7 except for aquaporin (data not shown).

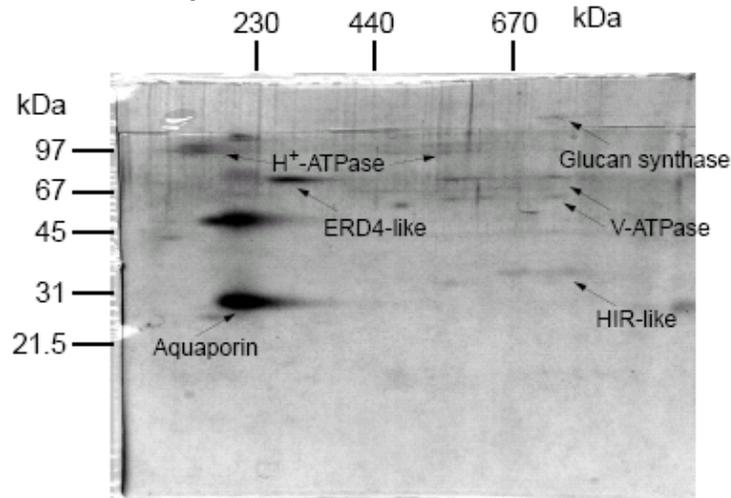
The HIR-like protein belongs to a superfamily related to stomatins and prohibitins that was found in *Zea mays* suggested to take a part in regulating channel activity (Nadimpalli et al., 2000). Contrary to the results obtained in the study presented here, a HIR-like protein identified through mass spectrometry has been short-term (1 day) up-regulated in abundance but long-term (3 days) down-regulated in *A. thaliana* upon cold treatment (Kawamura & Uemura, 2003). Sequence data was not disclosed in the Kawamura & Uemura (2003) study, and consequently, it may be two different proteins, both having sequence parts matching the same database reference. The sequence match in the study presented in this thesis was with *S. oleracea* material, matching sequences obtained from *O. sativa* and *Z. mays* (both 94 % identity). Sequence identity between K35 and the database *A. thaliana* HIR-like protein was 82 %.

### **BN/SDS-PAGE successfully resolved plasma membrane protein complexes in *A. thaliana***

BN/SDS-PAGE was also done on plasma membrane fractions from *A. thaliana*. Most prominent complexes observed for *S. oleracea* were seen also for *A. thaliana* (Fig 9) when studying several BN/SDS-PAGE gels. For all proteins but the higher mass oligomeric H<sup>+</sup>-ATPase (identified using mass spectrometry) in *A. thaliana*, migration similarity was regarded as confirmation of the same component. H<sup>+</sup>-ATPase was seen at 100 kDa as a

prominent component. A protein complex corresponding in migration to the aquaporin was detected as a tetrameric complex. A protein corresponding in migration to the ERD4-like protein and two proteins corresponding in migration to V-ATPase complexes were notable. Proteins corresponding in migration to the 30/35 complex, seen for *S. oleracea*, were not detectable in *A. thaliana*. A protein spot corresponding in migration to the HIR-like protein was seen in *A. thaliana*.

**Fig 9: BN/SDS-PAGE of plasma membrane fraction from *A. thaliana***



**Figure 9:** BN/SDS-PAGE resolution of plasma membrane fraction from *A. thaliana*. 800  $\mu\text{g}$  of protein was used as starting material. Proteins identified from *S. oleracea* are indicated by their corresponding positions in *A. thaliana*. Native molecular mass is shown on the horizontal axis, denatured molecular mass is shown on the vertical axis.

#### **Minigel BN-PAGE is possible for plasma membranes as a quicker way to make studies**

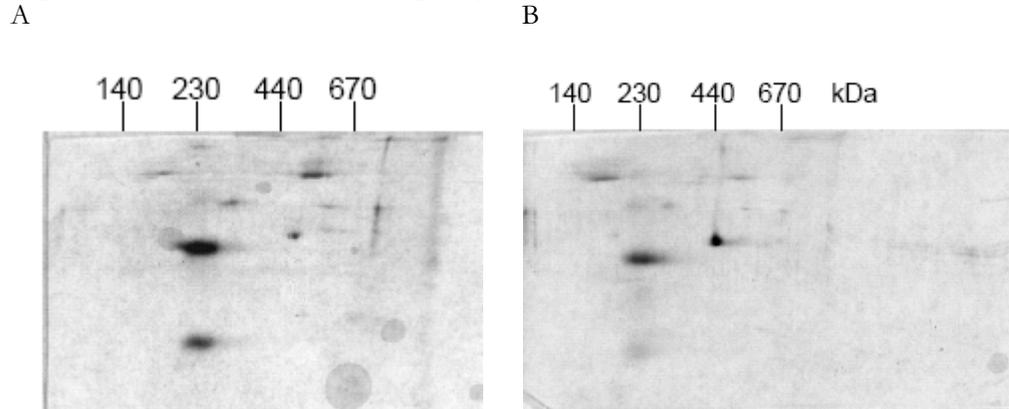
A practical disadvantage with BN/SDS-PAGE when done in a large system is the consumption of time and of large amounts of material. To overcome this, BN/SDS-PAGE was done in a scaled down version (Fig. 10), using a minigel system as in Brookes et al. (2002). The method was the same, but the protein amount loaded per lane was lower. The smaller system was able to resolve up to 150  $\mu\text{g}$  of membrane protein per lane before getting overloaded (data not shown). It is compatible with the standard methods of western blotting (aquaporin identified, data not shown) and mass spectrometric analysis (Brookes et al., 2002).

The result from down-scaling of the system to fit minigel format is seen in Fig 10. The procedure was similar to the procedure for gels of larger size, with a few exceptions explained in the legend to Fig 10.

As is seen from Fig 10, the decrease in total protein amount also reduces the detection limit, but the time consumption decreases dramatically (approximately by a factor 3 in

native dimension and a factor 4 in denaturing dimension) and so does the material amount required for resolution (approximately by a factor 5-10). The abundant proteins found in Fig 5 are all present in the gel presented in Fig 10. This technique would thus have advantages especially for western blotting analysis, where dye staining is less important.

**Fig 10: BN/SDS-PAGE in minigel system**



**Figure 10:** BN/SDS-PAGE using minigel system. Plasma membrane fractions (100 µg resolved) from either *A. thaliana* (A) and *S. oleracea* (B) was used. Native dimension was 4-14.5 % acrylamide. Native dimension temperature control was maintained by submerging the electrophoresis equipment into a beaker filled with ice and performing the electrophoresis in a cold room. The cathode buffer in the minigel system was exchanged for a cooled cathode buffer without Coomassie when the front had passed into the separation gel. The denaturation/solubilisation time in 1 % SDS/1% mercaptoethanol was shortened from 30 minutes to 10 minutes. Electrophoresis time was modified to finish the native dimension when sufficient band stacking was visually observed and denaturing dimension was electrophoresed until the front dye had reached the anode buffer, thereby decreasing the electrophoresis time but keeping the analytical checkpoints from a qualitative perspective.

### **An approach to improve comparison between treatments**

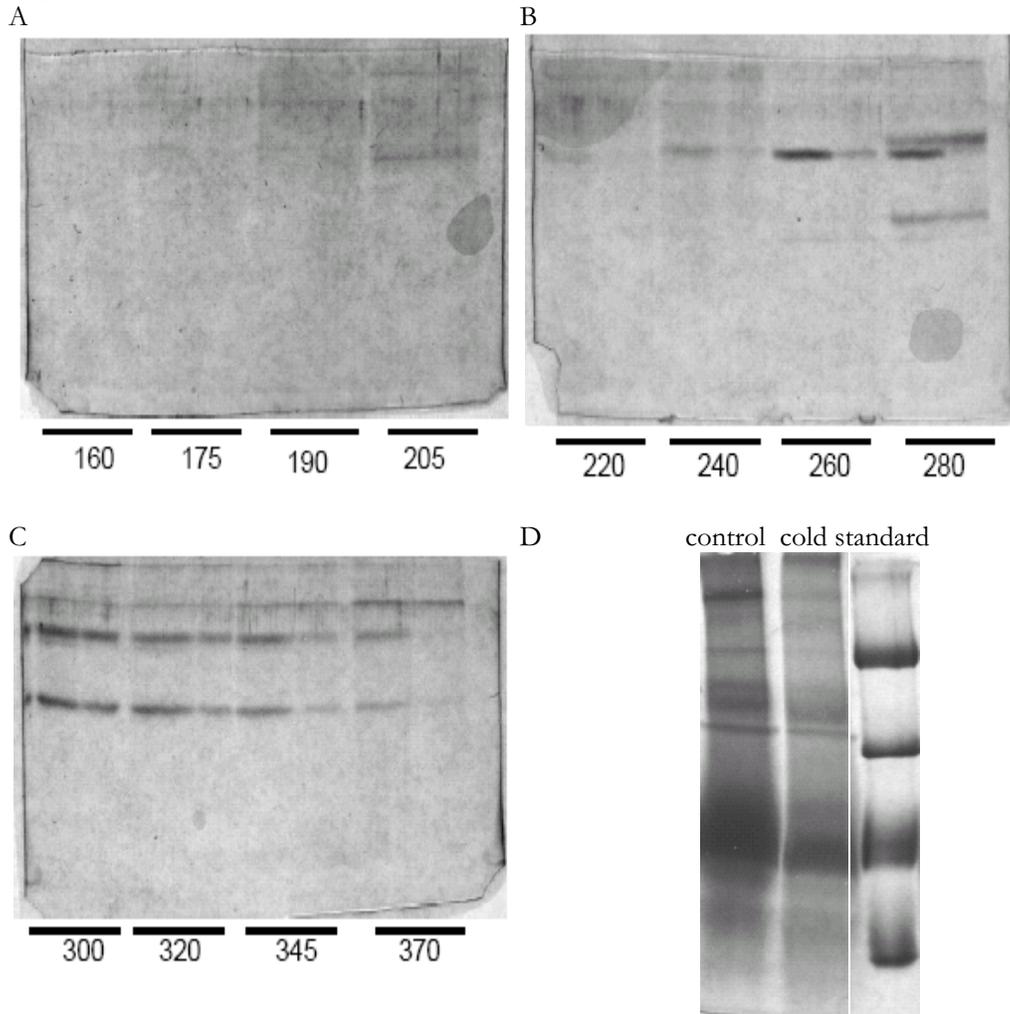
To improve the minigel approach and enable a better overlook on overlapping protein complexes in native and denaturing gel electrophoresis, a more comparative approach was employed. Horizontal slices were cut out from the native dimension lanes of electrophoresis using the sharp ends of a coverslip.

The slices were mounted between glass plates, embedded and resolved in a denaturing gel (Fig 11). This way it would be expected that an improved resolution with a banding pattern, similar to that obtained in traditional SDS-PAGE, would be achieved. At the same time, from the banding pattern it would be possible to qualitatively show changes in protein complex distribution or abundance when two different treatments, applied in adjacent native lanes, were compared.

The second dimension should provide evidence if proteins in ordinary BN/SDS-PAGE

are misinterpreted as part of the same complex but resolve into different slices in this comparative approach and thereby shown to be part of different complexes.

**Fig 11: A comparative approach of BN/SDS-PAGE**



**Figure 11:** Plasma membranes from *A. thaliana* untreated or cold-treated for 14 days were resolved in a native dimension of BN-PAGE (D). Slices of two adjacent lanes (the left in the pair being untreated, the right being cold-treated) were cut out after denaturation/solubilisation and mounted between glass plates for denaturing dimension SDS-PAGE. Slices were cut from below on the native gel and mounted from left to right, A-C, with increasing native molecular mass (indicated below the lanes in kDa). The apparent native molecular mass is indicated under each slice pair. No mass correction due to hydrophobicity is done for the slices. Each slice represents (4-14.5 % native gel, minigel system) a native resolution of 13-25 kDa per slice (1.5 mm slices) in the range 100-400 kDa native molecular mass, having a greater mass range at higher native masses due to the gradient gel. Native standard protein (11 D) is as in Fig 2.

As an example of the approach, plasma membrane fractions from *A. thaliana* were

compared with plasma membranes (*A. thaliana*) harvested after 14 days of cold treatment (Fig 11). Native molecular mass was calculated from the native dimension and hence each lane represents a slice of molecular mass span.

The components found in BN/SDS-PAGE analysis of *A. thaliana* plasma membranes are seen as bands at corresponding native molecular masses in Fig 11, indicating that the method can be compared to BN/SDS-PAGE described elsewhere. Of special interest is the finding that there is generally more protein loaded in the native control lane (Fig 11 D), but there is more aquaporin (identified by the subunit migration) in the 280 kDa slice for cold treated plasma membranes (Fig 11 B). Hence, the relative amount of aquaporin is higher in cold treated *A. thaliana* plasma membranes than in control plasma membranes.

It should be possible to tell whether streaking seen in denaturing dimensions using the BN/SDS-PAGE technique is due to unspecific aggregation (which would be expected to show up in the periphery of the horizontally cut out second dimension gels as discrete spots after horizontal cutting of slices) or due to uncertainty of native molecular mass (which would be expected to show up as a banding pattern over several horizontally cut out second dimensions) similar to a pattern that can be obtained in gel filtration (Poetsch et al., 1999; van der Hoorn et al., 2003).

The method outlined here has an increased usefulness in quantitative comparison and if detection of components of lower abundance is possible. Using the same amount of material but concentrating the sample so that more and narrower lanes is loaded onto the same gel would be an improvement and would also decrease the number of gels necessary to run in order to go through all the slices from a native gel. The application concentration is achieved if the protein complexes cut out horizontally would have their proteins electroeluted and embedded into agarose gel prior to second dimension mounting of the glass plates.

## PERSPECTIVES

### Perspectives

BN/SDS-PAGE is a robust method suited for studies of hydrophobic protein complexes in plant plasma membranes and can be combined with other methods. As a consequence of this, it may be a good starting point for more systematic investigations on plasma membrane protein composition, especially protein complex composition. This study indicates that as 2 % dodecyl maltoside solubilises complexes that are not solubilised that well at lower concentrations, it may be an advisable initial approach to use higher detergent concentrations unless there is particular reasons to do otherwise. The series with Triton X-100 shows the same relationship: that it may be advisable to use higher detergent concentrations than those often used. Together, these results give the conclusion that a high ionic strength and a high concentration of detergent should be the preferred general approach.

A good experimental setup would involve the study of abundant plasma membrane

protein complexes under different stresses, for example cold stress and salt stress; stresses that could generate global responses. Changes in composition, migration and prevalence in the abundant protein complexes would then possibly reveal structural changes of particular importance in the stress responses. A complex involved in several of these stresses would be regarded as a candidate for more general acclimation mechanisms.

One such stress response investigation would, as suggested earlier in the text, be to study cold acclimation of *S. oleracea* leaves in more detail and further resolve the protein complexes with BN/SDS-PAGE. If possible, the comparative approach suggested in this thesis could be used, using a native gradient gel of 10-15 % polyacrylamide, thus resolving only the complexes that have lower native molecular masses. Proteins that may contribute to the native molecular mass shift seen in *S. oleracea* after cold treatment (Fig 7) would be excised and subjected to mass spectrometry analysis. The study could then be including cold treatment of leaves from other species, such as the model plant *A. thaliana*, to conclude whether the shift is a generally occurring shift or if it is confined to *S. oleracea* which was studied here. The comparative approach could also be used to monitor subtle plasma membrane proteome differences due to other environmental conditions (salt stress, drought, etc.), age, differences between species or other relevant factors such as the effect on leaves by spraying with pesticides. The advantage with the approach is that the reproducibility and reliability is high as the slices are obtained from the same cut, and that uncertainty parameters such as differences in isoelectric focussing or mounting of lanes are excluded by the approach. To lower the detection limit, the protein complexes from the first dimension may possibly be electroeluted as described in Novotná et al. (1999) and loaded in thinner wells in the second dimension.

When the different abundant protein complexes and components have been studied and detected, other more sensitive detection methods such as silver staining could be employed to obtain a more refined pattern of complexes and subunits. Many complexes involved in responses such as signal perception and signal transduction exist in low copy numbers and would not be expected to be detected until more refined studies are conducted. Probably, these studies would involve antibody detection or strongly concentrating preparations.

Investigations of components of lower abundance could benefit from various prefractionations done in order to use more material as starting material, discarding fractions containing highly abundant complexes before gel analysis. Thus, higher amounts of proteins of interest can be loaded. This would increase the resolution of the complexes of lower copy number, except for those co-eluting with abundant complexes in prefractionation. Abundant complexes in the system investigated would already be known. An approach here is to use different gradients or size-exclusion chromatographic techniques combined with concentration methods.

An alternative approach for comparison of treatments would be differential labelling with staining dyes such as Cy2, Cy 3 and Cy5, shown to work for IEF/SDS-PAGE (Tonge et al., 2001). Different samples are then be labelled with different dyes, pooled and subjected to BN/SDS-PAGE. Provided that the dyes are compatible with the Coomassie G-250 and are neither excluding the Coomassie nor are excluded by the Coomassie, fluorescence patterns corresponding to the different dyes would indicate differences introduced upon

plant treatment. However, this approach would require development and evaluation as new parameters not tested for compatibility with BN/SDS-PAGE are introduced into the system.

Changes in protein complex abundance may or may not be correlated to a change in expression. Using western blotting of SDS-PAGE gels or similar would tell the investigator whether the expression pattern follows the protein complex presence during a particular stress. The study could be combined with a study involving real-time PCR to monitor the expression of a treated and an untreated sample. This correlation or lack of correlation would indicate whether appearance or disappearance of a protein complex is due to synthesis of the protein components or due to recruitment from or to an existing pool of monomeric components.

An interesting question that could be addressed using BN/SDS-PAGE is the nature of lipid rafts. If detergent-resistant membrane domains could be isolated and a mild detergent able to solubilise protein complexes within the lipid raft, comparison of lipid rafts derived from different sources could give an increased knowledge on the nature of the lipid rafts. Such a detergent should be mild, at least partly exchangeable to Coomassie Brilliant Blue G-250 and have properties that makes it suitable for solubilising a cholesterol- and sphingolipid-rich environment (Simons & Ikonen, 1997), possibly with a longer hydrophobic stretch than detergents used for solubilising detergent-soluble domains (McIntosh et al., 2003). An analysis could concern the association between GPI-anchored proteins or other components to the actin cytoskeleton which has been suggested from energy barrier studies in lipid rafts (Suzuki & Sheetz, 2001).

BN/SDS-PAGE appears to work well for the study of biological membrane protein complexes. As for all methods, there are some aspects to be aware of in order to use the method properly, though. Lack of results using the BN-SDS/PAGE technique would have a limited number of probable reasons. The method outlined here is adapted for integral plasma membrane protein complexes. Any component so loosely associated to the membrane that it is disconnected upon pelleting of the membranes will be lost. If the component is not solubilised using the amino caproic acid buffer combined with the solubilising detergent, it will also not be detected. Protein interactions that are too weak to allow solubilisation with preserved protein complex integrity will also lead to failure in detecting the physiological native complex with BN/SDS-PAGE. Also, if proteins are degraded in or lost from the gel strips during solubilisation/reduction steps, it will not be detected. Finally, components of an abundance below the detection limit of the detection system will also not be detected. Fortunately, for studies on mitochondrial membranes and chloroplast membranes, all previously known major complexes have been detected. This may be an indication that most hydrophobic complexes of sufficient abundance can be studied using BN/SDS-PAGE and future progress in refining the method and methods used in tandem will certainly increase the investigation possibilities to include the study of components of lower abundance.

The method is possible to use with equipment that is to be considered more or less standard equipment, i.e., that is most probably already present in most laboratories. The essential machinery includes only basic laboratory equipment found in laboratories working with proteins. The investigations benefit considerably from the access to a mass

spectrometer, but results on a smaller scale can be obtained even without this more expensive equipment. Computer software for quantification of gel scans and immunoblotting results also facilitate the interpretations. Hence, the basic method is a tool not restricted to laboratories with a generous funding situation and this should help spreading the use and speeding up further elaboration of the technique, though of course a good funding situation increases the array of opportunities offered by the technique.

## SVENSK POPULÄRVETENSKAPLIG SAMMANFATTNING

Proteomforskningen, alltså forskningen om helhetsbilden av alla de proteiner som finns i cellerna, har under senare år utvecklats till ett eget forskningsfält. Det har funnits kvar vissa svårlösta problem för proteomforskningen, främst när det gäller proteiner som sitter i biologiska membran. Det yttersta av cellens membran är plasmamembranet och det är detta membrans proteinkomplex, det vill säga funktionellt interagerande proteiner, som jag har studerat i mitt arbete.

I avhandlingen har jag visat att en metod som kallas BN/SDS-PAGE (en separationsmetod där interaktionerna mellan proteiner bibehålls) kan användas för att studera sammansättningen av proteinkomplex i växters plasmamembran. Metoden gör det möjligt att studera de nativa (i sina funktionella enheter) hydrofoba proteinkomplexen, det vill säga de membranproteiner och associerade proteiner som bildar enheter i cellen. I metoden separeras proteinkomplexen först nativt, därefter separeras komplexens beståndsdelar.

De mest förekommande plasmamembranproteinkomplexen kunde kartläggas med hjälp av BN/SDS-PAGE. Dessa komplex utgörs av vattenkanalproteiner, protonpumpar, proteiner som bildar cellväggsliknande sockerpolymerer och proteiner som sannolikt bidrar till att bibehålla cellernas struktur vid miljöpåfrestningar. I avhandlingen visades hur en förändring av ytermiljön såsom köldbekämpning påverkar dessa proteinkomplex, liksom hur behandling med olika salter kunde påverka komplexen. Ytterligare en variant av separationsmetoden presenterades också.

Metoden är en av få som gör det möjligt att studera vilka proteiner som bildar funktionella enheter i membranet och avhandlingen visar att metoden fungerar på plasmamembran från flera olika växtslag. Förhoppningsvis kan metoden få en ökad spridning i framtiden, då den tycks mycket lovande och underutnyttjad, samtidigt som den inte är särskilt resurskrävande utan kan användas med apparatur som vanligen redan finns i laboratorier där proteinforskning bedrivs.

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# APPENDIX

## Protein complexes of the plant plasma membrane resolved by Blue Native PAGE

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### Abstract

With the characterisation of the total genomes of *Arabidopsis thaliana* and *Oryza sativa*, several putative plasma membrane components have been identified. However, a lack of knowledge at the protein level, especially for hydrophobic proteins, have hampered analyses of physiological changes. To address whether protein complexes may be present in the native membrane, we subjected plasma membranes isolated from *Spinacia oleracea* leaves to blue-native polyacrylamide gel electrophoresis (BN-PAGE). BN-PAGE is well established in the separation of functional membrane protein complexes from mitochondria and chloroplasts but a resolution of the PM protein complexes from eukaryotic cells have previously not been reported. Using this method, protein complexes from *Spinacia oleracea* PM could be separated, including the highly hydrophobic aquaporin (apparent molecular mass 230 kDa), a putative tetramer of H<sup>+</sup>-ATPase, and several less abundant complexes with apparent masses around or above 750 kDa. After denaturation and separation of the complexes into their subunits in a second dimension (SDS-PAGE), several of the complexes were identified as hydrophobic membrane proteins. Large amounts of protein (up to 1 mg) can be resolved in each lane, which suggests that the method could be used to study also low-abundant protein complexes, e.g. under different physiological conditions.

**Abbreviations:** ACA, amino caproic acid; BN, Blue Native; Brij 58, polyoxyethylene 20 cetyl ether; DoMa, n-dodecyl-β-D-maltoside; IEF, isoelectric focusing; PAGE Polyacrylamide Gel Electrophoresis, PM, plasma membrane; SDS, sodium dodecyl sulfate.

### Introduction

The plasma membrane (PM) defines the outer barrier of the cell through which ions, water and metabolites must pass under strict regulatory control. It is also the site for primary events in several signalling cascades and houses some of the machinery for synthesis of extracellular components (Larsson et al. 2001). With the characterisation of the total genome of *Arabidopsis thaliana* and *Oryza sativa*, several putative PM components have been identified based on similarities with corresponding components in organisms

other than plants. However, knowledge at the protein level is lacking, except for a few examples, e.g. the H<sup>+</sup>-ATPase and the aquaporin. Proteome investigations with highly purified PM have been conducted in joint efforts between several laboratories, where solubilised membrane proteins have been separated in two dimensions by isoelectric focusing (IEF) and SDS-PAGE, and identified by protein sequencing (Santoni et al. 1998). However, using these methods, most of the proteins identified were peripheral to PM. This prompted specific investigations on the conditions used in solubilisation and separation to also recover hydrophobic proteins. The combination of membrane washing with using zwitterionic detergents improved solubilisation (Santoni et al. 1999), but in general, hydrophobic proteins are poorly solubilised and may precipitate during IEF. Though information now has been obtained with 2-D IEF/SDS-PAGE for selected proteins by using specific detergents and strong denaturing conditions (Molloy 2000), the method is not optimal developed for membrane proteome studies (van Wijk 2001). Furthermore, the method does not provide information on the functional units, i.e., whether the proteins are monomeric or part of protein complexes, including their own oligomeric state.

Electrophoretic separation of functional membrane protein complexes was originally demonstrated for bovine heart mitochondrial membranes using blue native polyacrylamide gel electrophoresis, BN-PAGE (Schägger and von Jagow 1991), and later used for analyzing chloroplast and mitochondrial membrane protein complexes from plants (Jänsch et al. 1996, Kügler et al. 1997, Rasmusson et al. 1994). In 2-D BN/SDS-PAGE, solubilised proteins are separated first by native and then by denatured molecular mass (Schägger and von Jagow 1991). In the first dimension, the native proteins are given a negative charge by binding Coomassie G-250, and separated according to native size on gradient gels. The resolved proteins are subsequently denatured in the gel and constituent polypeptides are resolved in a second dimension according to denatured mass. The resolved polypeptides can thereafter be identified by immunoblotting or, after tryptic digestion, by mass spectrometry and sequenced peptides compared with sequences published in databases. The method has not previously been used for resolution of isolated plasma membranes from eukaryotes. It has been used to investigate specifically labelled and immunodetected proteins such as the yeast H<sup>+</sup>-ATPase during export from ER to PM (Lee et al. 2002), disease resistance proteins in microsomal fractions of tobacco (Rivas et al. 2002 ab), and of animal PM glycine transporters heterologously expressed in oocytes (Horiuchi et al. 2001). Though a total protein resolution has not been reported, this still indicates that PM proteins are intrinsically competent for analysis by this technique. BN-PAGE has also been used to confirm the interaction between 14-3-3 protein and the C-terminus of the H<sup>+</sup>-ATPase upon fusicoccin treatment of plant PM (Oecking and Hagemann 1999). In this case, water-soluble tryptic protein fragments were separated and resolution of the intact proteins was not reported. Thus, investigations on the whole PM protein complex proteome have not been performed up to now. Here, we show that several high molecular mass PM protein complexes and their subunits can be resolved in isolated PM using 2-D BN/SDS-PAGE after minor modifications of the original protocol (Schägger and von Jagow 1991). Several of the proteins are transmembrane and their oligomeric state can thus be estimated. These include well known and abundant proteins such as the H<sup>+</sup>-transporting ATPase, the aquaporin and the V-ATPase, but also components that have been connected to stress responses.

## Materials and Methods

### *Plant culture and membrane isolation*

Spinach (*Spinacea oleracea* cv. "Carambole") was grown at 20°C, (16 h light, 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , 50-55 % humidity), for 4-5 weeks in the green house. Spinach was chosen for ease of getting large amounts of highly purified PM. A microsomal fraction microsomal fraction was obtained from a leaf homogenate as described (Larsson et al. 1994). The microsomal fraction was subjected to phase partitioning to yield one fraction highly enriched in right-side-out plasma membrane vesicles (PM), and another fraction depleted in PM but enriched in intracellular membranes as described (Larsson et al. 1994). The latter contains a diversity of membranes from the microsomal fraction except the PM. The PM corresponds to only ca three per cent of the microsomal fraction (not shown). The final fractions were suspended in 0.33 M sucrose, 10 mM N-[2-hydroxyethyl]piperazine-N'-[ethanesulfonic acid] (Hepes), pH 7.5, and stored at  $-80^{\circ}\text{C}$  until use. Protein was determined (Bearden 1978) using BSA as standard.

The data presented below were obtained with membrane fractions isolated from the same leaf homogenate, to better allow qualitative comparisons. The BN/SDS-PAGE pattern with PM was obtained at least three times with similar results, using PM isolated at different occasions.

### *Membrane washing*

For each lane of BN-PAGE, the membrane fractions (800  $\mu\text{g}$  protein) were diluted with 0.33 M sucrose, 10 mM Hepes, pH 7.5 and supplemented with 1 mM EDTA, 1 mM phenyl methyl sulfonyl fluoride, 1  $\mu\text{M}$  pepstatin A, 3  $\mu\text{M}$  N-(trans-Epoxy succinyl)-L-leucine 4-guanidinobutylamide (E-64; Sigma Chemicals Co., St Louis, MO, USA) (final concentrations) including either of the following: a) for control membranes: no additives; b) for Brij washed membranes: 0.5 % (w/w), Brij 58 (Sigma Chemicals Co., St. Louis, MO, USA), protein to Brij ratio 1:10, c) for NaCl-washed membranes: 0.5 % (w/w) Brij 58, 1 M NaCl and d) for KI washed membranes: 0.5 % (w/w) Brij 58, 0.5 M KI. After 30 min on ice the membranes were pelleted at  $186,000 \times g_{\text{max}}$  for 60 min and resuspended in a small volume of ACA buffer (1.5 M amino caproic acid, 300 mM BisTris, pH 7.0).

### *Membrane solubilisation and 2-D BN/SDS-PAGE*

For solubilisation, n-dodecyl- $\beta$ -D-maltoside (DoMa) was added to resuspended fractions to a final concentration of 2 % (w/w). After incubation on ice for 15 min, insoluble membrane material was pelleted at  $100,000 \times g_{\text{max}}$  for 30 min. To the supernatant, Coomassie G-250 (Serva Blau G-250, Serva Biochemica, Heidelberg, Germany) was added to a final concentration of 0.7 % (w/v). The samples (protein corresponding to 800  $\mu\text{g}$  of the initial membrane fraction, see above) were loaded onto a 4-18 % gradient BN-PAGE (separating gel ca 11 cm) and subjected to native electrophoresis at  $4^{\circ}\text{C}$  (Jänsch et al. 1996). Electrophoresis was initially carried out at 30 V and increased to 500 V (approximately 10 mA) when the stained front had passed into the separation gel. Molecular mass markers were from Amersham Pharmacia (product number 17-0445-01, Amersham BioSciences, Uppsala, Sweden). Electrophoresis was carried out overnight at  $4^{\circ}\text{C}$ . After this step, the lanes were cut out, washed in 1 % (w/w) SDS, 1 % (v/v)  $\beta$ -mercaptoethanol for 30 min and rinsed with distilled water (Jänsch et al. 1996). The separation in the second dimension was performed in a 10 % Tris/Tricine SDS-gel with a 6 % stacking gel (Jänsch et al. 1996). Molecular mass markers were from BioRad (product

numbers 161-0304 and 161-0303, BioRad Laboratories, Hercules, CA, USA). Electrophoresis was started at 30 V and increased to 40 mA (80 V -120 V) when the front had passed into the separation gel, then run until the front reached the anode buffer (approximately 800 Vh).

After electrophoresis, the gels were either stained with Coomassie R-250 (BioRad Laboratories, Hercules, CA, USA) analyzed by immunoblotting (see below).

#### *Coomassie R-250 staining*

After SDS-PAGE, gels for Coomassie staining were put into 0.1 % Coomassie R-250 (BioRad Laboratories, Hercules, CA, USA) in 50 % methanol, 7 % acetic acid, destained in 25 % methanol, 7 % acetic acid, and dried between cellophane sheets.

#### *Immunoblotting*

Electrotransfer was carried out with a BioRad Transblot apparatus set at 200 V for 2 h (BioRad Laboratories, Hercules, CA, USA) using Immobilon™-P membranes (Millipore Corporation, Bedford, MA, USA). The membrane was blocked with 5 % fat free milk powder in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, washed and incubated in primary antibody against 28 A aquaporin (Johansson et al. 1998) overnight at 7°C. After incubation with secondary antibody, detection was obtained using the Enhanced Chemiluminescence protocol (BioRad Laboratories, Hercules, CA, USA).

#### *Mass spectrometry*

Proteins, separated in the second dimension, were excised from gel and cleaved with trypsin by in-gel digestion. Peptide analysis was performed by electrospray ionisation mass spectrometry according to (Wilm et al. 1996) on a Q-tof using Masslynx software (Micromass, Manchester, UK). Sequence homology searches were carried out using the BLAST program (Altschul et al. 1997) or an MS-BLAST program (Gish 2003, Shevchenko et al. 2001).

#### *Sucrose gradient centrifugation and SDS-PAGE*

To test the homogeneity of the PM fraction, isolated PM (ca 3 mg) was applied on top of a 10 ml 0.5-1.8 M sucrose gradient (10 mM Hepes, pH 7.5; 0.1 mM EDTA, 1 mM dithiothreitol) and centrifuged for 2 hours at 85,000 x  $g_{av}$  using a SW28.1 swing-out rotor at 4°C. Fractions of 2 ml were collected from below using a peristaltic pump. The fractions were stored at -80°C until use. Thawed samples from the sucrose gradient were solubilised in 67.5 mM Tris, 10 % glycerol, 6 % SDS, 2.5 % mercaptoethanol (final concentrations) at room temperature for 5 min and separated by SDS-PAGE (Laemmli 1970) at 9 % polyacrylamide using the BioRad minigel system (BioRad Laboratories, Hercules, CA, USA).

## **Results**

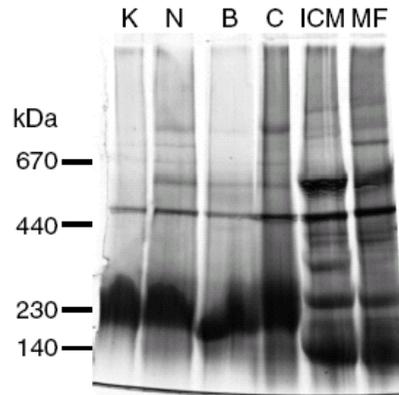
#### *Separation of protein complexes in isolated plasma membranes*

Isolated spinach leaf PM was solubilised at different protein concentrations with different concentrations of digitonin, Triton X-100 or DoMa and protein complexes separated by BN-PAGE. Of these detergents, 2 % (w/v) DoMa gave the sharpest separation (not shown) and was therefore chosen in the subsequent experiments. In the protocol for mitochondria (Schägger and von Jagow 1991). 750 mM ACA was used for enhancing solubilisation. We found that a higher ACA concentration during solubilisation (1.5 M)

greatly improved separation in the first dimension (not shown). This concentration was therefore used in the subsequent experiments.

Membranes were solubilised with DoMa and subjected to BN-PAGE. Prior to solubilisation, the PM fraction had been washed in different ways to release peripheral proteins. The initially purified PM fraction consists of membrane vesicles that expose their original outside out, e.g. are right side-out (Larsson et al. 1994). Treating PM vesicles with Brij 58 results in that these vesicles are turned inside-out (Johansson et al. 1995) so that enclosed or loosely attached components will be released and removed upon recentrifugation. Brij 58-turning in combination with NaCl washing will further deplete inside-out PM vesicles in electrostatically associated peripheral proteins.

Fig 1



**Fig. 1: Blue native gel electrophoresis of solubilised plasma membranes isolated from spinach leaves.** The lanes show native protein complexes in the microsomal fraction (MF), intracellular membranes (ICM) and PM (C), as well as PM turned inside-out with Brij 58 alone (B), or with additionally NaCl (N) or KI (K) present to release peripheral proteins. The migration of molecular mass markers thyroglobulin, ferritin, catalase and lactate dehydrogenase are given in kDa at the side. After separation, the gel was stained with Coomassie R-250. For further details, see Materials and Methods.

Several native high molecular mass components were visible after BN-PAGE separation of the solubilised microsomal fraction, intracellular membranes and PM in the first dimension. The general native protein pattern of all PM fractions differed substantially from the patterns in the other two other fractions (Fig 1). The latter were dominated by thylakoid proteins, where bands around 140 kDa and 600 kDa probably are the light harvesting complex IIb and Photosystem I+ light harvesting complex I as judged by chlorophyll content and their reported migrations in these gel systems (Kügler et al. 1997). In PM, a dominating protein complex was found at ca 230 kDa, and this component was less evident in the microsomal fraction and in the intracellular membranes. Other protein complexes present in PM were at 600 and 650 kDa, and at least two more complexes apparently specific to PM were found above 670 kDa, the highest molecular mass standard used. Besides these, several protein complexes of

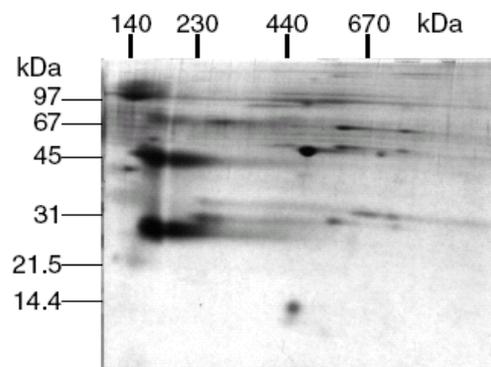
intermediate sizes were seen in PM. A protein complex around 500 kDa was found in all membrane fractions.

The inside-out PM obtained by Brij 58 washing, with or without NaCl, contained less protein compared to control (mainly right-side out PM), as seen from the decreased band intensities (the same initial protein amount was used for the respective membrane washes and what is seen in the lanes reflect the protein that is membrane-associated and pelletable after these washes). If the chaotropic salt KI was used, an even larger loss was obtained, and only the major complexes could be distinguished (not shown). To obtain more information on the identity of the protein complexes, separation of them into their constituents was done in a second dimension SDS-PAGE (Figs 2-6).

*Separation of protein complexes into their respective subunits in the second dimension*

Denaturation of control PM protein complexes and separation of these in the second dimension is shown in Fig. 2. The dominating species is still the 230 kDa complex which here separated into a 28 kDa and a 45 kDa components. Both forms were specifically recognised by an antibody against aquaporin 28A (not shown). The components probably corresponded to aquaporin monomers and dimers, their presence being due to incomplete denaturation prior to the second dimension. A third component of the aquaporin corresponding to a trimer was detected at 75 kDa in some gels (not shown).

Fig. 2



**Fig. 2: Two-dimensional BN/SDS-PAGE of control PM.** Protein complexes of control PM (Fig. 1, lane C) were resolved in two dimensions by BN-PAGE (horizontal) and SDS-PAGE (vertical). Location of native and denatured protein markers are given in kDa above and at the side, respectively. After separation, the gel was stained with Coomassie R-250. For further details, see Materials and Methods.

A second dominating component of the first dimension had a native apparent molecular mass around 140 kDa and a denatured MW around 97 kDa. This protein was enriched in PM and depleted in intracellular membranes, as compared to the microsomal fraction from which these two fractions were isolated (Figs 2, 3 and 4). Other distinct PM components were from the high molecular mass complexes at 600 and approximately 750 kDa (Fig. 2), the latter being above our largest standard. These

complexes separate in the second dimension into several subunits, i.e., at ca 30 kDa, 60 kDa and 70 kDa. These components were also visible in the microsomal fraction and to a lesser extent in the intracellular membranes (Fig 3 and 4). The complex at 500 kDa separated into two spots in all fractions, having molecular masses agreeing with the large and small subunits of Rubisco. This was later proven by sequencing of the larger subunit (Table 1).

Fig. 3

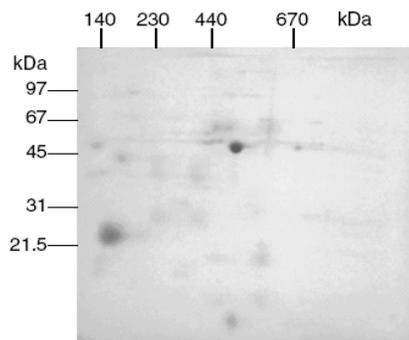
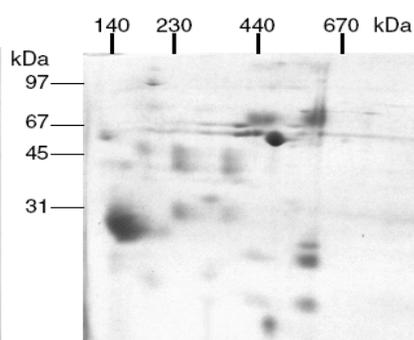


Fig. 4



**Fig. 3: Two-dimensional BN/SDS-PAGE of a microsomal fraction.**

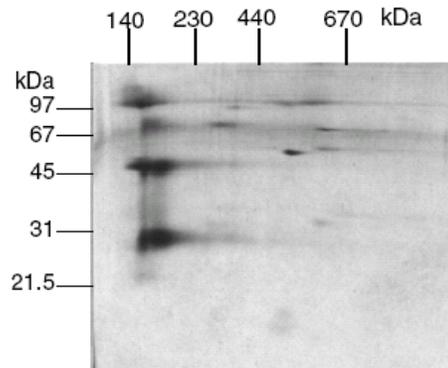
Protein complexes of the microsomal fraction (Fig. 1, lane MF) were subjected to second-dimension SDS-PAGE. Otherwise as for Fig. 2.

**Fig. 4: Two-dimensional BN/SDS-PAGE of intracellular membranes.**

Protein complexes of intracellular membranes (Fig. 1, lane ICM) were subjected to second-dimension SDS-PAGE. Otherwise as for Fig. 2.

Treatment of PM fractions with Brij 58 so that the cytosolic side of the membrane proteins were exposed, not only resulted in some loss of total protein (Fig 1), but also in qualitative changes. One example was the disappearance of a component with a denatured molecular mass of about 55 kDa and belonging to a complex of around 700 kDa (cf Fig 2 and Fig 5). This component was found in the microsomal fraction (Fig 3) and less so in the intracellular membranes (Fig 4) and might represent a soluble component that had been trapped in the PM vesicles and again released as these were turned inside-out with Brij 58. Alternatively, it represents a loosely attached peripheral PM protein, since detergents at low (<CMC) concentrations can strip membranes of loosely attached components. It is known that high salt concentrations also may cause release of peripheral proteins from membranes (25, 26), but treatment of inside-out PM vesicles with NaCl did not appear to result in any further release of protein (Fig 6).

Fig 5



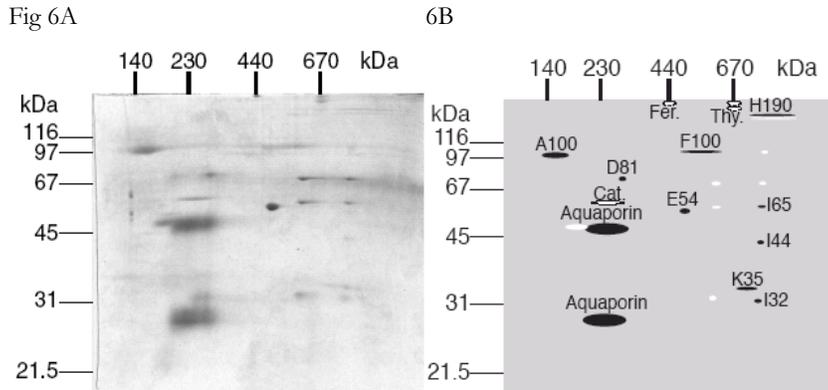
**Fig. 5: Two-dimensional BN/SDS-PAGE of Brij 58-washed PM.** Protein complexes of Brij 58-washed PM (Fig. 1, lane B) were subjected to second-dimension SDS-PAGE. Otherwise as for Fig. 2.

#### *Mass spectrometry*

The identity of the major high-molecular mass components in PM was established by mass spectrometric analysis of tryptic digests of protein spots. This was done for the PM fraction that had been washed by Brij 58 in the presence of 1 M NaCl (Fig 6; Table 1). The H<sup>+</sup>-ATPase (denatured molecular mass at 97 kDa) appeared at more than one location, i.e., in complexes with apparent molecular masses around 140 (spot A100) and 600 kDa (spot F 100), respectively. Several other polypeptides were found to comigrate in the native dimension, but it was difficult to determine with certainty whether any of these were associated to the H<sup>+</sup>-ATPase complex. The two locations instead most likely represent monomeric and tetrameric units of the 97 kDa component.

The spot D81 had a native molecular mass close to 300 kDa and a denatured molecular mass at 81 kDa (Fig 4, Table 1). It showed 85-100 % sequence similarity in three sequenced peptides with a 81.9 kDa expressed protein (supported by cDNA) from *A. thaliana* belonging to a family of hypothetical eukaryotic transmembrane proteins of unknown function. An *A. thaliana* cDNA fragment corresponding to the 81.9 kDa protein was identified as early-responsive to dehydration and named *erd4* (Kiyosue et al. 1994). No other components were seen to comigrate with this the complex.

Interestingly, the vacuolar membrane V-ATPase was recovered in PM (Fig 4, Table 1) as a complex with molecular mass of approximately 750 kDa. Spots I32, I44 and I60 were identified as subunits E, C and B, respectively, of the catalytic domain (V<sub>1</sub>). A 71 kDa component comigrating in the native dimension is probably identical to subunit A of the same complex. A faint band was also seen at 100 kDa. Subunits A, B, and E but not C were also found as part of a smaller mass form of 600 kDa. Due to the proximity to spot F100, it was not possible to judge whether the 100 kDa spot was also present in the 600 kDa form of the V-ATPase. Both molecular mass forms and their subunits could also be seen in the other membrane fractions (cf Figs 2, 3 and 4).



**Fig 6: Two-dimensional BN/SDS-PAGE of NaCl-washed PM.** Protein complexes of Brij 58- and NaCl-washed PM (Fig. 1, lane N) were subjected to SDS-PAGE. (A) Coomassie stained gel. (B) Scheme of (A), sequenced polypeptides denoted in black, other distinct bands in white. Thyroglobulin (Thy.), ferritin (Fer.) and catalase (Cat.) are visible as internal standards and marked by dashed lines. Otherwise as for Fig. 2.

**Table I. Protein identification by mass spectrometry.**

Spot nr	Peptide sequence*	Native complex (kDa)	Name or description	Amino acid length	Accession nr (database)	Species	% identity/ % similarity**
A100	KIDQSSLTGESLPVK	140	Plasma membrane proton ATPase-like	956	At5g62670 (AGI)	A. thaliana	100/100
F 100	KIDQSSLTGESLPVK	550-600	Plasma membrane proton ATPase-like	956	At5g62670 (AGI)	A. thaliana	100/100
D 81	KVNEIWEEL DTWTVEQPEPR KVYVPSF	300	Expressed protein	724	At1g30360 (AGI)	A. thaliana	77/100 82/82 85/100
E 54	ENVNSQPFMR, KTFQGGPPHGIQVER	500	Rubisco large subunit	475	CAB88737 (EMBL)	S. oleracea	100/100 100/100
H 190	FPDEWENFLER RDVGLNQIALFEG	> 750	Putative glucan synthase	784	At2g36850 (AGI)	A. thaliana	100/100 100/100
I 32	KANEISVAAEIEEFNTR ELVVQSLLR	> 750	V-ATPase E subunit	229	Q41396 (swissprot)	S. oleracea	100/100 100/100
I 44	RYGLPPNFLASVLPVSLK	> 750	V-ATPase C subunit	375	At1g12840 (swissprot)	A. thaliana	72/72
I 65	KYTTVQFTGEVLK RTVSGVAGPLVLLDK	> 750	V-ATPase B subunit	488	Q40078 (swissprot)	H. vulgare	100/100 100/100
K 35	RDSVLNFSENVPGTSAK REQIQAYVFDVIR, RDGLLQGG	>750	Hypothetical 31.4 kDa protein	286	Q9ZRU2 (EMBL)	C. arietinum	94/94 92/92 100/100

\* All analyzed peptides were assumed to be preceded by Arg (R) or Lys (K) since trypsin cleaves after these amino acids. R and K are marked in bold in the peptide sequences where these amino acids are also found in the database sequences.

\*\* for the proposed peptide sequence judged from mass spectra

Two more PM-components were identified by mass spectrometry, one of them being putative glucan synthase (spot H190) present in a complex with native mass

larger than 750 kDa and associating with a polypeptide of slightly lower denatured molecular mass. In the intracellular membranes no component corresponding to glucan synthase was found as judged by migration (Fig 3), consistent with an exclusive PM location. Finally, spot K35, showed 88-100 % identity in three sequenced peptides to the deduced protein sequence (molecular mass 31.4 kDa) of *Zea mays* hypersensitive-induced response (HIR) genes belonging to a gene family involved in plant disease responses. They are part of the protein superfamily including also prohibitins and stomatins (Nadimpalli et al. 2000) and suggested to be involved in ion channel regulation. The spinach HIR component was not detected in the intracellular membranes, indicating a PM-specific location. The distinct broader location of K35 in the native dimension varied somewhat between individual gels relative to the 750 kDa V-ATPase complex, indicating that they are not associated in a single complex.

## Discussion

We here show that BN-PAGE successfully could be used to characterise integral membrane protein complexes of plant PM. Of the tested solubilisation conditions, 2 % sodium dodecyl maltoside gave best resolution by BN-PAGE in agreement with similar studies of thylakoid protein complexes (Kügler et al. 1997). Elevation of the ionic strength during solubilisation also greatly improved resolution, including solubilisation of notoriously difficult proteins like aquaporin.

Blue-native PAGE has been shown to be generally non-destructive with respect to protein complexes (Schägger and von Jagow 1991). It has been successfully employed to plant mitochondrial (Jänsch et al. 1996) as well as chloroplast systems (Kügler et al. 1997, Poetsch et al. 2000) and the protein complexes are separated in their enzymatically active form (Schägger and von Jagow 1991, Poetsch et al. 2000). Therefore, although a higher amino caproic acid concentration is needed for efficient solubilisation of PM, it is likely that the method could preserve most plant PM complexes intact as well. This we see an example of in the detection of a high molecular mass form of V-ATPase (Fig. 6). The possibility to apply a general approach for membrane proteins is very attractive, as hydrophobic proteins are often difficult to solubilise for IEF-PAGE, and hence a basic method that allows some degree of comparison between different membrane types is worth considering as a standardised tool.

With BN/SDS-PAGE, information is obtained on the oligomeric state of membrane proteins. However, the amount of dye and detergent that is bound by hydrophobic protein is larger than the amount bound by available marker proteins. Therefore, the apparent molecular mass should be larger than the size calculated from sequence or the sum of the denatured masses of the subunit(s). Dye and detergent binding raised the apparent molecular mass by a factor of almost 2 in a study of several membrane transport proteins (Heuberger et al. 2002). With this in mind, the detection of the H<sup>+</sup>-ATPase, having an established molecular mass of 97 kDa, at a native molecular mass of 140 kDa and ca 600 kDa, should correspond to monomeric and tetrameric ATPase, respectively. Similarly, the highly hydrophobic aquaporin with a monomer molecular mass of 28 kDa, may be a tetramer, even though the complex was found at 230 kDa. This agrees with that the aquaporins have been found as tetramers in other studies (Kamsteeg et al. 2000).

The glucan synthase migrated here as part of a complex with an apparent molecular mass above 750 kDa. This glucan synthase is part of the family of callose synthases which are transmembrane proteins involved in the synthesis of extracellular  $\beta$ -

1,3-glucans. Two callose synthase complexes could be distinguished in solubilised membrane extracts of *Vigna aconitifolia* (Hong et al. 2001 ab). In these studies, several other enzymes were found to associate with callose synthase and suggested to be part of very large callose synthase complexes as judged by sedimentation data. A loosely connected, and possibly variable presence of other bound proteins is consistent with the less distinct migration of glucan synthase (Fig. 6). The presence of glucan synthase in the PM was expected since this agrees with biochemical information (Widell and Larsson 1990), and in fact, its activity is one of the more absolute markers for this membrane in plants (Widell and Larsson 1990).

The presence of two molecular mass forms of the V-ATPase reminds of the mitochondrial  $F_0F_1$ -ATP synthase, which normally is found both as intact complex and as  $F_1$ -domain alone upon 2-D BN/SDS-PAGE analysis (Jänsch et al. 1996). The V-ATPase is the main protein complex of the vacuolar membrane (tonoplast), comprising up to a third of the total vacuolar membrane protein content. Therefore, minor contaminants of vacuolar membranes in the PM preparation may result in that the V-ATPase will be detected also here. Many studies have indicated that vacuolar membranes in most cases are lighter than PM and that they therefore can be separated using density gradients (Widell and Larsson 1990). However, when spinach leaf PM was subjected to gradient centrifugation, no fractions specifically enriched in the V-ATPase (e.g. A and B subunits) were distinguished and most proteins coeluted from the gradient, indicating that the PM preparation was homogeneous with respect to membrane vesicles (not shown). Using immunocytochemistry with pea cotyledons, V-ATPase has been found in PM as well as in the vacuolar membrane (Ratajczak 2000), indicating that the V-ATPase may not be completely specific for the latter.

The V-ATPases are composed of several polypeptides in two domains, the peripheral domain  $V_1$  and the membrane-integral domain  $V_0$  (Ratajczak 2000). Reported subunits of the plant  $V_1$  head are: A (63-72 kDa) and B (52-60 kDa), and of the  $V_1$  stalk: C (37-52 kDa), D (30-42 kDa), E (27-32 kDa), F (13 kDa) and G (13 kDa). Reported subunits of the plant  $V_0$  domain are: c (16-20 kDa), M39 (32-36 kDa) and M115 (95-115 kDa). With Coomassie stain, subunits A, B, E were visible in both V-ATPase mass forms and additionally subunit C in the larger form (Fig 6, Table 1). A subunit D may however be masked by the K35 HIR protein. Asymmetries in stoichiometry and Coomassie binding may prevent the detection of additional subunits. The gels indicate a faint 100 kDa polypeptide (see e.g. Fig. 2) in the 750 kDa complex. This is a molecular mass similar to the  $V_0$  M115 which could indicate that the 750 kDa mass form represents intact V-ATPase. The size also agrees with a calculated 730 kDa for the whole enzyme (Ratajczak 2000). It is less evident what form of V-ATPase that results in a molecular mass species of 600 kDa. The size is too large to reflect only  $V_1$ , but too small to reflect the intact complex minus subunit C (and possibly D). Thus, it probably is a complex containing the  $V_1$  subunit and some of the  $V_0$  components. Its distinct band in BN-PAGE suggests that the subcomplex is relatively stable, i.e. what is seen is not a transient intermediate in a continual loss of subunits.

The location of the HIR protein, i.e. the 31.4 kDa protein, referred to as K35 in Table 1 and Fig. 5, needs a special comment. It seems to be located mainly in PM and could not be washed off by NaCl treatment. It also seems to contain only one subunit, i.e., at ca 35 kDa. Stomatin, a distantly related membrane component in red blood cells forms multimeric complexes of about 9-12 similar subunits (Snyers et al. 1998). The C-terminal region of stomatin has a high  $\alpha$ -helical content (Stewart 1997) and in C-

terminal-truncated stomatin no oligomerisation was found, suggesting that the  $\alpha$ -helical (i.e. hydrophobic) part was important in oligomerisation (Snyers et al. 1998). A high oligomeric state could be the case also for the K35 spinach protein, since the apparent molecular mass of the complex is above 750 kDa. Closely related proteins of the HIR family in maize were predicted to have a  $\alpha$ -helices near the C-terminus (Nadimpalli et al. 2000). A HIR protein was also detected in *Lotus japonicus* peribacteroid membranes (Wienkoop and Saalbach, 2003). In contrast to our results, this HIR protein was part of a complex with several other components, e.g. a P-ATPase as well as different soluble proteins. The molecular mass of the native complex was not given, but it was higher than that of another complex containing e.g. the V-ATPase. This suggests that it is part of a different complex compared to that described here for spinach PM, although the peribacteroid membrane in part originates in PM.

Resolution of the native protein complexes has to our knowledge not been made for isolated PM before, neither from plants nor from other eukaryotes. Since up to one mg protein can be separated simultaneously, it should be possible to detect also minor PM components and identify them using Western blotting or mass spectrometry, which is especially important since many putative PM proteins should be expected to occur only in low amounts. Besides, with BN-PAGE transmembrane PM proteins are efficiently isolated and with gentle agents. This opens up possibilities to functionally characterise protein-protein interactions in PM during a developmental process or as a response to stress.

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