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Protein pre-fractionation in detergent–polymer aqueous two-phase systems for facilitated proteomic studies of membrane proteins

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Abstract

Pre-fractionation of a complex mixture of proteins increases the resolution in analytical separations of proteins from cells, tissues or organisms. Here we demonstrate a novel method for pre-fractionation of membrane proteins by a detergent-based aqueous two-phase system. Membrane proteins are strongly under-represented in proteomic studies based on two-dimensional electrophoresis (2-DE). As a model system, we have isolated mitochondria from the yeast *Saccharomyces cerevisiae*. Mitochondrial proteins were fractionated in an aqueous two-phase system consisting of the polymer poly(ethylene glycol) and either of two commonly used non-ionic detergents, Triton X-114 or dodecyl maltoside (DDM). Soluble proteins partitioned mainly to the polymer phase while membrane proteins were enriched in the detergent phase, as identified from one-dimensional electrophoresis (1-DE) and/or 2-DE followed by mass spectrometric analysis. Pre-fractionation was further enhanced by addition of an anionic detergent, sodium dodecyl sulfate, or a chaotropic salt, $NaClO_4$, and by raising the pH in the system. The two-phase system pre-fractionation was furthermore combined with an alternative two-dimensional high-resolution separation method, namely ion-exchange chromatography and 1-DE. By this approach a larger number of membrane proteins could be identified compared to separation with conventional 2-DE. Thus, pre-fractionation of complex protein mixtures using the aqueous two-phase systems developed here will help to disclose larger proportions of membrane proteins in different proteomes.

Keywords: Aqueous two-phase systems; Proteomics; Surfactants; Proteins

1. Introduction

Membrane proteins, such as receptors, transporters and channels, are of great interest because of their participation in various important cellular mechanisms such as signalling and molecular trafficking across the membrane. Therefore, these proteins serve as interesting target molecules in pharmaceutical research. A major challenge with membrane proteins are that their hydrophobic properties make them more difficult to separate compared to soluble proteins. One of the most frequently used separation technique for proteome analysis is two-dimensional electrophoresis (2-DE) followed by identification with mass spectrometry (MS) analysis. Proteomic analysis of membrane proteins encounters problems mainly for the following reasons: (1) hydrophobic proteins have low solubility in the isoelectrical focusing (IEF) step of 2-DE [1]. This has been the subject of substantial research and progress has been made with new detergents, chaotropes, solubilisation techniques and pre-treatments of the sample [2–9]. Although it has been possible to identify membrane proteins with up to 12 transmembrane α-helixes by 2-DE–MS methodology [10] most membrane proteins are still not detected at all by this technique. The number of identified membrane proteins from 2-DE was compared with a subcellular fractionation step followed by one-dimensional

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electrophoresis (1-DE) by Galeva et al. [1]. Only three microsomal membrane proteins could then be identified using different types of 2-DE, while 22 were identified from the combination of fractionation and 1-DE. (2) Due to a lack of tryptic cleavage sites in the membrane spanning domains of the proteins (i.e. lysine and arginine), digestion with trypsin often results in large, hydrophobic peptides that are not readily detected by MS and hydrophobic peptides are under-represented and poorly resolved in MS analyses. Thus, a smaller number of peptides originating from loops connecting hydrophobic segments and containing tryptic digestion sites are available, which will lower the chance for identification. To increase sequence coverage of hydrophobic peptides a combination of proteases and cyanogen bromide and addition of the detergent octyl-β-glucopyranoside has been used to obtain a more complete set of peptides found in the spectra [11,12]. (3) Many membrane proteins of low abundance will disappear in the bulk of high-abundance proteins, which in majority are soluble proteins. The number of detectable membrane proteins on 1-DE/2-DE can therefore be increased by finding efficient methods to enrich membrane proteins and to remove contaminating water-soluble proteins. Efforts have been made to improve pre-fractionation of complex samples [1,2,9]. One approach is to use organic solvents. A combination of chloroform and methanol has previously been used to differentially extract membrane proteins from chloroplasts [13]. Blonder et al. used a combination of washing isolated membranes with carbonate-buffer and organic solvent extraction to increase the number of identified hydrophobic proteins [14]. Differential extraction of proteins based on detergent solubility is an alternative to the organic solvents for enrichment of different protein classes in different fractions [15]. Centrifugal sucrose gradient fractionation for preparation of membranes is another alternative pre-fractionation method that was applied to isolated human mitochondria prior to 1-DE [16].

Cloud point extraction, also called temperature induced phase separation, of the non-ionic detergent Triton X-114 [17–19] is a method similar to the aqueous two-phase partitioning described in this work, for fractionation of membrane proteins from soluble proteins. When temperature is increased, a solution of Triton X-114 phase separates into one detergent enriched phase and one aqueous phase. Cloud point extraction in Triton X-114 solution in combination with hydroxyapatite column chromatography has previously been applied to fractionate membrane proteins prior to 2-DE–MS analyses. It was shown that a cytosolic protein (HSP90) and a membrane protein, with one transmembrane domain (VLDL receptor), with similar isoelectric points and molecular weights were successfully separated using cloud point extraction and 2-DE–MS analyses [20].

We demonstrate here a method for enrichment of membrane proteins prior to proteomic analysis. The overall strategy is schematically outlined in Fig. 1 and, a protein extract of mitochondria from *Saccharomyces cerevisiae* (bakers yeast) was used as model system. Isolated mitochondria

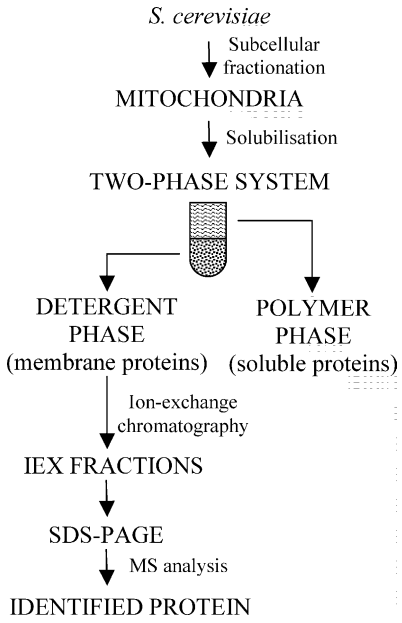

Fig. 1. Methodology outline. Mitochondria from *S. cerevisae* were isolated by differential centrifugation. The isolated mitochondria were fractionated in a detergent–polymer aqueous two-phase system formed by addition of polymer and salt to the detergent solubilised mitochondrial membranes. Membrane proteins partition to the detergent (bottom) phase and the bulk of soluble proteins to the polymer (top) phase. The detergent phase was further fractionated with ion-exchange chomatography and fractions were analyzed with 1-DE. Bands were identified with MS analysis.

from *S. cerevisiae* were used mainly for two reasons. (1) Intact mitochondria contain both membrane proteins and soluble proteins. (2) The entire genome of *S. cerevisiae* is sequenced and readily available on-line.

We present a novel aqueous two-phase system consisting of a commonly used detergent in membrane protein research, *n*-dodecyl-β-D-maltoside (DDM) [21–26], and poly(ethylene glycol) (PEG), a common polymer with a wide range of use in biotechnology [27]. In our laboratory it has previously been found that mixtures of DDM–PEG and Triton X-114–PEG result in a phase separation [28] forming two aqueous phases. A detergent–polymer two-phase system shows similar phase behaviour to a polymer–polymer two-phase system, e.g. the much used dextran–PEG system [29]. In detergent–polymer systems, the detergent micelles can be regarded as the second polymer [30,31]. Thus, a range of non-ionic detergents, at concentrations above the critical micellar concentration (CMC), has been shown to phase separate with water-soluble polymers, e.g. dextran and PEG [28]. Detergent–polymer aqueous two-phase partitioning has also been shown to be a mild technique for obtaining a larger fraction of biological active membrane protein compared to cloud point extraction [32]. The system should have great potential for the extraction of large membrane protein complexes, since there is no steric hindrance as in chromatographic separation techniques. Main reasons for mildness and pre-fractionation advantages of the described system compared to cloud point extraction are:

(1) phase separation is achieved by a different mechanism i.e. the polymer induces phase separation and formation of a detergent micelle rich phase and a polymer rich phase and no temperature increase is needed [28,33]. Therefore, proteins can be separated even at 0 °C with a wide range of mild, non-ionic detergents [28] which is advantageous for protein stability. (2) Polymers are also known for stabilizing proteins [27]. (3) Detergent–polymer aqueous two-phase systems have a robust phase separation behaviour [33]. Thus, a large loading capacity towards complex biological materials such as membranes enables the system to efficiently enrich membrane proteins into a small detergent phase. The system is, consequently, also suitable for detecting novel membrane protein interaction partners [34].

The described method for pre-fractionation of membrane proteins by detergent/polymer two-phase systems could be combined with either conventional 2-DE or another two-dimensional separation step, namely ion-exchange chromatography followed by 1-DE (Fig. 1). With this method several membrane proteins not readily detected in unfractionated mitochondria or in 2-DE, could be readily detected. This technique offers a potential for detection of a larger proportion of membrane proteins from many different proteomes.

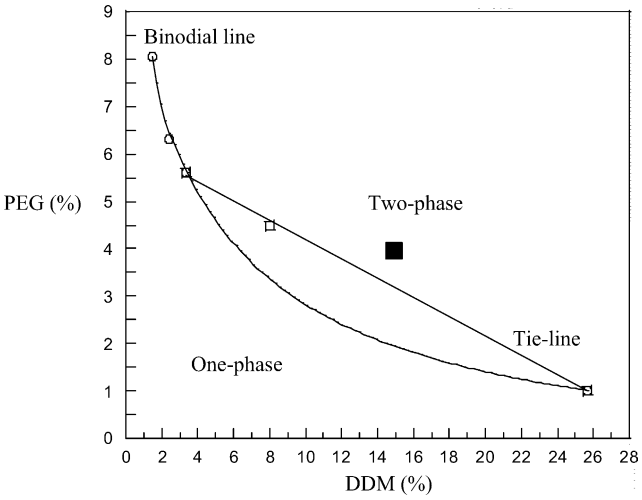

Fig. 2. Phase diagram of a dodecyl maltoside–poly(ethylene glycol) aqueous two-phase system at 4 °C. The phase boundary between the one-phase region and the two-phase region is called the binodial curve. Phase composition of the phases can be determined from the tie line. Along a tie line the phase composition is constant but the volume ratio between the phases can be altered. The system used in the experiments is marked out in the figure (■).

2. Experimental

2.1. Phase diagram of PEG–DDM two-phase system

When detergent and polymer are mixed in concentrations above the phase boundary (binodial curve) two distinct phases are formed at equilibrium and a phase diagram can be determined (Fig. 2). Binodial curve and phase compositions were determined according to [28]. To determine the binodial curve, PEG (molecular mass: 40 000) and DDM were mixed in concentrations where two phases were formed. The total mass of the systems was 0.5 g and included buffer [10 ml 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.0]. All measurements were made in a cold room (4 °C). Buffer was added to the systems in small aliquots until the system no longer separated into two phases and one homogenous phase was obtained. When no phase separation could be detected after centrifugation at 1800 × *g*, 5 min, systems were considered to be monophasic. Phase compositions of the systems, i.e. tie lines, were determined with polarimetry (using the digital polarimeter model AA-10, Optical Activity, London, UK) and refractive index (using a differential refractometer, Carl Zeiss, Oberkochen/Württ, Germany) according to Albertsson [35]. Only the binodial was determined for the Triton X-114–PEG system (results not shown).

2.2. Preparation of mitochondria from S. cerevisiae

Commercially processed *S. cerevisae* was purchased from a grocery store. Pressed yeast (400 g) was suspended in 200 ml mitochondria isolation buffer (MIB) containing 0.6 M mannitol, 20 mM HEPES–KOH, pH 7.4, using a Dounce homogenizer. The mitochondria were isolated according to McAda et al. [36]. Glass beads (450–600 μm) and yeast suspension were added to a Beadbeater (BioSpec Products, Bartlesville, OK, USA). The suspension was homogenized 5 × 20 s with 1 min rest on ice between each treatment, pooled and centrifuged twice, 3500 × *g* for 10 min. To the supernatant complete protease inhibitor cocktail tablet (Boehringer Mannheim, Germany) dissolved in MIB was added according to manufacturers instructions. The supernatant was centrifuged for 20 min at 17 000 × *g* 4 °C to pellet the mitochondria. The pellet was washed once and the centrifugation step was repeated. The mitochondrial pellet was resuspended in MIB (with protease inhibitor cocktail) and stored in −80 °C. Protein concentration of the suspension was measured to ∼15 mg/ml by BCA assay [37].

2.3. Solubilization of mitochondria and two-phase partitioning

Isolated mitochondria (70 μl, total protein concentration: 15 mg/ml) were incubated in 16.6% (w/w) DDM or 5% (w/w) TX-114, in a total volume of 200 μl, for 15 min on a rocker platform in 4 °C for solubilization. The samples were centrifuged at 100 000 × *g* for 45 min to remove unsolubilized material. After solubilization the supernatant was transferred to the two-phase systems. All two-phase systems were created according to [28]. Glass tubes (diameter 6 mm, length 50 mm) were used and the total mass of each system was 0.5 g. In the case of the DDM–PEG system the components were mixed from stock solutions to get final

concentrations of 15% (w/w) DDM [from 40% (w/w) stock solution] and 4% (w/w) PEG 40 000 [from 25% (w/w) stock solution] (Fig. 2). The systems also included 10 mM HEPES, pH 7.0, or 10 mM Tris–HCl, pH 8.0 or 9.0, and to the mixed micelle systems 3 mM sodium dodecyl sulfate (SDS) was added. In the case of TX-114–PEG, systems were mixed to obtain final concentrations of 11% (w/w) TX-114 [from 30% (w/w) stock solution] and 4% (w/w) PEG 40 000 [from 25% (w/w) stock solution], 10 mM Tris–HCl pH 9.0, 100 mM $NaClO_4$. All components were weighed in and thoroughly mixed and the tube was placed on a rocker platform for phase equilibration at 4 °C for 15 min. To speed up phase separation the tubes were centrifuged at 1600 *g* in a tabletop centrifuge for 10 min at 4 °C The phases were isolated using a Pasteur pipette and analysed for total protein concentration with the BCA method. To determine the partitioning of proteins between phases in a system a partition coefficient (K value) can be calculated. The *K* value is defined as the concentration of the analyte in the top phase divided by its concentration in the bottom phase.

2.4. Ion-exchange chromatography

Detergent phase from the DDM–PEG two-phase system (total mass 3 g) at pH 9.0, 3 mM SDS was diluted to 7.0 ml with 20 mM Tris–HCl, pH 8.0, 0.25% DDM (buffer A) and applied to a Resource Q 1 ml anion-exchange column (Amersham Biosciences, Uppsala, Sweden) equilibrated with buffer A. Elution was performed by applying a gradient of 0–50% 20 mM Tris–HCl, pH 8.0, 0.25% DDM, 2.0 M NaCl (buffer B) over 40 column volumes, followed by a step elution with 100% buffer B over 5 column volumes. Eluted proteins were detected at 280 nm and collected in 1 ml fractions.

2.5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

The isolated phases were diluted with the appropriate buffer and prepared for SDS-PAGE using PlusOne SDS-PAGE Clean-Up Kit (Amersham Biosciences, Uppsala, Sweden) according to the manufacturers instructions. NuPAGE Bis-Tris pre-cast gradient gels 4–12% from Novex (San Diego, CA, USA) were used to perform the SDS-PAGE with 50 mM 3-(*N*-morpholino)propane sulfonic acid (MOPS) and 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) as running buffer. Electrophoresis conditions were set to 200 V, 125 mA for 60 min . The gels were stained in Colloidal Coomassie [38] and destained in water. Fractions from ion-exchange chromatography were prepared for 1-DE using PlusOne SDS-PAGE Clean-Up Kit. Electrophoresis of the fractions was performed on an 8–18% gradient ExceGel (Amersham Biosciences) gradient-gel according to manufacturers instructions. The gels were stained with GelCode (Pierce, Rockford, IL, USA) and destained in water.

2.6. 2-D PAGE

Isoelectric focusing (IEF) was carried out using Immobiline DryStrips, 7 cm, pH 3–10 non-linear (NL), IPG buffer pH 3–10 NL, IPG cover fluid (Amersham Biosciences). The rehydration solution contained 7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 100 mM freshly added dithiothreitol (DTT), 2% (v/v) IPG buffer and some grains of bromophenol blue. The samples were loaded by including them in the rehydration solution for at least 10 h at room temperature. The IEF was performed with an IPGphor system (Amersham Biosciences) as follows, 500 V for 30 min, 1000 V for 30 min and 8000 V until at least 19000 V h was reached. The strips were equilibrated according to manufacturers instructions in 50 mM Tris–HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 65 mM freshly added DTT and a few grains of bromophenol blue. Strips not directly transferred to the second dimension were stored at −80 °C. NuPAGE Bis-Tris pre-cast gradient gels 4–12% from Novex (San Diego, CA, USA) were used to perform the SDS-PAGE step of the second dimension. The strip was placed on top of the SDS-PAGE gel and sealed with 0.5% (w/v) agarose (including a few grains of bromophenol blue) and run in 50 mM MOPS at 40 mA, 200 V for 15 min , followed by 80 mA, 200 V for 75 min. The gels were stained in Coomassie Brilliant Blue R-250 and destained in 20% methanol (Fig. 5).

2.7. In-gel digestion and peptide extraction

In-gel digestion and peptide extraction was performed as according to Shevchenko et al. [39]. The colloidal coomassie stained protein bands of interest were cut out of the gel using a scalpel and put in Eppendorf tubes. The gel pieces were rinsed in water for 5 min. The water was removed and four times the volume of the gel pieces of acetonitrile (ACN) was added to shrink the gel pieces for 15 min. The liquid was removed and the gel pieces were dried in a SpeedVac concentrator (Savant, Farmingdale, NY, USA). To reduce the proteins 40 μl 10 mM DTT in 100 mM NH_4HCO_3 was added and incubated for 30 min at 56 °C. The pieces were spun down and the liquid was removed. The acetonitrile-shrinking step was repeated. To alkylate the proteins 40 μl 55 mM iodoacetamide in 100 mM NH_4HCO_3 was added and incubated at room temperature in darkness for 20 min. The gel pieces were washed twice with 100 mM NH_4HCO_3 for 15 min. The ACN shrinking step was repeated again. The gel pieces were rehydrated in digestion buffer containing 50 mM NH_4HCO_3, 5 mM $CaCl_2$ and 12.5 ng/μl of sequencing-grade trypsin (Promega, Madison, WI, USA) on ice for 45 min. Digestion buffer without trypsin was added to cover the pieces and keep them wet during enzyme cleavage. The samples were left at 37 °C overnight. The supernatant was collected. To extract the peptides from the gel particles 25 mM NH_4HCO_3

was added and incubated for 15 min at 37 °C with shaking. Then acetonitrile (twice the volume of the gel particles) was added and the incubation step repeated. The gel particles were spun down and the supernatant was collected and pooled with the supernatant from the overnight incubation. A volume of 40 μl 5% formic acid was added and the tubes were incubated for 15 min at 37 °C ACN (twice the volume of the gel particles) was added and incubated for 15 min in 37 °C with shaking. The gel particles were spun down and the supernatant was pooled with the rest. The samples were concentrated to a volume of approximately 10 μl using a SpeedVac concentrator.

2.8. Mass spectrometry

The extracted and concentrated peptides were desalted using small pre-packed reversed-phase columns, ZipTips (Millipore, Bedford, MA, USA), according to manufacturers instructions. The peptides were eluted directly on the matrix-assisted laser desorption ionization (MALD) target with a solution saturated with the matrix α-cyano-4-hydroxy cinnamic acid. For MALDI time-of-flight MS a Voyager-DE STR (Per-Septive Biosystems, Framingham, MA, USA) mass spectrometer was used to obtain the mass spectra at positive reflector mode. 150 shots were collected for each spectrum and the ions were accelerated at 20 kV. In the case of electrospray ionization quadmpole time-of-flight (ESI-QTOF) elution from the ZipTips was made with 10 μl 50% ACN, 0.1% trifluoroacetic acid (TFA) Mass spectrometry and tandem mass spectrometry were performed on a quadropole time-of flight hybrid, Q-TOF (Micromass, Manchester, UK), equipped with a nano-ESI source. A 1 μl aliquot sample was loaded and MS data were acquired over 200–1800 *m/z* for 1 s.

2.9. Database searches

MALDI-TOF-MS spectra were processed using the software Data Explorer and database searches were carried out by submitting peptide mass fingerprints from the processed spectra to the ProFound search engine (http://www.prowl.rockefeller.edu/cgi-bin/ProFound) using the NCBI or SwissProt database. A hit was considered to be a positive identification when the probability was 1 and the so-called estimated *Z*-value exceeded 1.65. Taken under consideration all parameters given to the database search engine, a hit with a *Z*-value of 1.65 is considered to be a correct identification with a 95% confidence. The theory and basis of how the normalized probability of the protein identified and *Z*-score is calculated is described by Zhang et al. [40]. Some hits that did not reach a *Z*-value of 1.65 were still considered to be positive identifications if the probability was 1, the molecular mass of the protein had a good correlation with the position on the gel and at least five peptides matched with a minimum of 0.1 Da mass accuracy.

ESI-QTOF-MS–MS data was processed using MassLynx 3.4, exported in a SEQUEST format and submitted to a database search, using MASCOT (Matrix Science, http://www.matrixscience.com). The following parameters were used: peptide mass tolerance: ±1 Da; fragment mass tolerance: ±0.3 Da max missed cleavage: 3. The database searched was an in-house generated non-redundant database based on SwissProt and Trembl and the identification was considered to be positive when the MOWSE score exceeded 70 (this value is depending on the size of the database used) [41].

3. Results and discussion

3.1. Using increased pH and addition of $NaClO_4$ or SDS to enrich membrane proteins in the detergent phase of detergent/polymer aqueous two-phase systems

A phase diagram for the DDM–PEG system is presented in Fig. 2. In general membrane proteins are found to partition to the detergent phase and water-soluble proteins to the polymer phase in these systems [28]. To get a more efficient removal of water-soluble proteins from the membrane proteins in the detergent phase the chaotropic salt $NaClO_4$, or anionic detergent, SDS, was added to the system to create an electrostatic potential difference between the phases. This can be exploited as an advantage in protein partitioning by varying the pH of the system since it further enhances the removal of soluble proteins by the following mechanism. The ClO_4^- ions prefer the detergent phase making it slightly negatively charged [42] while SDS form so called mixed micelles with non-ionic detergents [43]. This introduces negative charges to the detergent phase and by adjusting pH of the system, the net charge of proteins can be altered. Thus, negatively charged soluble proteins will be directed towards the polymer phase because of electrostatic repulsion from the SDS incorporated in the non-ionic detergent micelles or the ClO_4^- ions enriched in the detergent phase [28,44,45]. Here SDS was added to 3 mM, which is well below the CMC of SDS. Thus, SDS does not form micelles but will be included in the DDM micelles with non-ionic detergent and membrane lipids.

In Fig. 3 we have calculated the *K* values based on the total protein concentration in the phases. In the systems where no SDS was added the *K* values were all around 0.3, i.e. the proteins are predominantly partitioned to the detergent phase regardless of pH. Upon addition of SDS to the Systems the *K* value was not significantly influenced at pH 7.0, but the effect could be seen in the system at pH 8.0 where the *K* value was approximately 1, which means an equal distribution of proteins between the phases. At pH 9.0 the value was approximately 2 showing that a majority of the proteins were partitioned to the polymer phase. Thus, the total protein value was changed from ∼0.3 to ∼2 by addition of SDS and change of pH. This

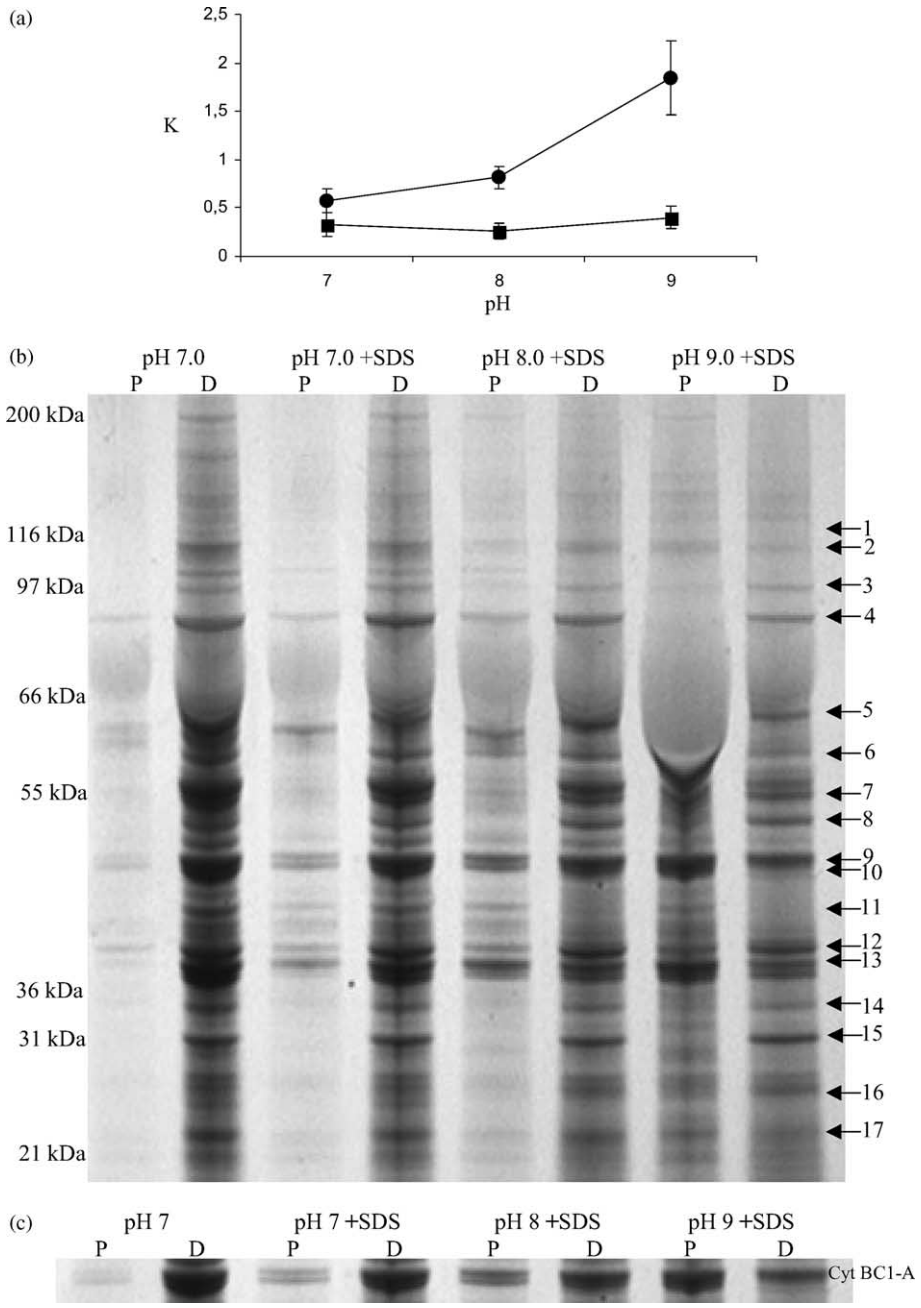

Fig. 3. Protein distribution between the polymer phase and the detergent phase. (a) K = partitioning coefficient ($C_{top\ phase}/C_{bottom\ phase}$). Here the K values are calculated from the total protein concentration of the phases when pH was increased in the absence of SDS (■) and when SDS was added to the system (●). (b) SDS-PAGE after two-phase partitioning of isolated yeast mitochondria which shows loading of equal volumes withdrawn from each of the phases. P: Polymer phase. D: detergent phase. Identified proteins are numbered and listed in Table 1 and show a number of examples of soluble proteins shifting from the detergent phase to the polymer phase with increasing pH and addition of SDS. (c) Highlighted region of M_r 53 000–45 000 in Fig. 3b. Example of increased resolution of a membrane protein subunit (Cyt BC1-A) in the detergent phase by removal of four water-soluble proteins to the polymer phase (Table 1) in the system at pH 9.0 and in the presence of 3 mM SDS. All systems were composed of 4% (w/w) PEG, 15% (w/w) DDM and 10 mM buffer (pH 7.0 HEPES; pH 8.0 and 9.0, Tris–HCl). SDS was added to a concentration of 3 mM.

can be explained by the fact that at this pH more proteins have a negative net charge, leading to a pronounced change in distribution of soluble proteins towards the polymer phase due to repulsion from negatively charged micelles in the detergent phase [44,46]. Membrane proteins were retained in the detergent phase (analysed with 1-DE, Fig. 3b, Table 1) due to the hydrophobic interaction with the detergent micelles. Addition of a small amount of SDS (3 mM) and changing the pH from 7 to 9 do not significantly affect the phase diagram of the system, indicating that the system is robust and the phase behaviour is not easily disturbed.

Table 1
Proteins from DDM–PEG two-phase partitioning with increasing pH and addition of SDS followed by 1-DE (Fig. 3) identified with MALDI-TOF-MS

Number	Protein name	SwissProt ID[a]	p*I*	TMD[b]
1	Ykl215cp	P28273	6.3	1
2	Phosphofructokise β	P16862	6.1	
3	Glycogen phosphorylase	P06738	5.4	
4	Aconitase	P19414	8.4	
5	Succinate dehydrogenase, flavoprotein subunit	Q00711	7.2	MA
6	Acetyl CoA hydrolase	P32316	6.3	
7	ATP synthase, α-subunit	P07251	9.2	MA
	Aldehyde dehydrogenase	P00360	6.3	
8	ATP synthase, β-subunit	P00830	5.5	MA
9	Cytochrome BC1 complex subunit A (Cyt BC1-A)	P07256	6.8	MA
	Citrate synthase	P00890	8.2	
	Enolase	P00924	6.2	
10	Phosphoglycerate kinase	P00560	7.8	
	Isocitrate dehydrogenase	P28834	8.9	
11	Ygr086p	P53252	4.5	
12	Branched chain amino acid biosynthesis, Ilv5	P06168	9.2	
13	Glyceraldehyde 3-phosphate dehydrogenase	P00358	8.4	
14	ADP/ATP translocator	P18239	9.9	6
15	Voltage-dependent anion-selective channel (VDAC)	P04840	9.9	16
16	ADP-ribosylating factor binding protein	P38817	6.1	1
17	Phosphoglycerate mutase	P00950	9.1	

All systems were composed of 4% (w/w) PEG, 15% (w/w) DDM and 10 mM buffer (pH 7.0; HEPES, pH 8.0 and 9.0; Tris–HCl), 3 mM SDS (not in reference system); temperature 4 °C. p*I*: isoelectric point.

[a] Accession number in the SwissProt database.

[b] Transmembrane domains (TMDs) are predicted from the TMHMM 2.0 server (http://www.cbs.dtu.dk/services/TMHMM-2.0/) in the SwissProt or CYGD databases, except in the case of VDAC which consists of transmembrane β-strands and not α-helixes.

Thus, addition of a small amount of SDS to the detergent–polymer aqueous two-phase system is crucial to optimise removal of soluble proteins from membrane proteins and achieve an efficient pre-fractionation. Modifying the two-phase system in this manner does not seem to affect the mildness of the method. A number of subunits originating from membrane protein complexes were enriched in the detergent phase, e.g. ATP-synthase α and β subunits (Table 1), which do not have any transmembrane domains. This suggests that membrane protein complexes are kept intact in the two-phase system. The proteins identified as subunits attached to transmembrane subunits of membrane protein complexes are here called membrane associated (MA, Table 1). Why is SDS, an ionic detergent known to be a strong protein denaturant in its monomeric form, not causing protein denaturation? The reason is that when mixed with non-ionic detergent the CMC of SDS is lowered due to formation of mixed micelles [43,44]. Thus, the monomer concentration of SDS is reduced which is favourable for protein stability. This was shown previously by Sivars et al. [28] where enzyme activity of the model proteins tested was retained in the presence of SDS and the non-ionic detergent $C_{12}EO_5$.

To identify and investigate the putative membrane proteins enriched in the detergent phase, and to show that the soluble proteins were removed from the detergent phase to the polymer phase the protein content in each of the isolated phases from the DDM–PEG system was analysed with 1-DE (Fig. 3b) followed by identification by MS analyses (Table 1). The shift in protein distribution between the phases caused by addition of SDS and increased pH (Fig. 3a) was confirmed by 1-DE. A number of proteins observed in the detergent phase of the reference system at pH 7.0 (no SDS) disappear in the detergent phase of systems at pH 8.0 and 9.0 in the presence of SDS to instead appear in the corresponding polymer phase.

A closer investigation of the various bands (Fig. 3b) by MALDI-TOF-MS showed that the resolution of membrane proteins was increased by removal of abundant soluble proteins, as pointed out in the highlighted area (Fig. 3c). In Fig. 3c the majority of proteins are found in the detergent phase of the reference system at pH 7.0 and the bands 9 and 10 are badly resolved and here no identifications could be made. However, in the detergent phase of the system at pH 8.0 in the presence of SDS, band 9 was detected and identified as a mixture of the core subunit A from the membrane protein cytochrome BCl complex together with two soluble proteins, enolase and citrate synthase, while band 10 was identified as a mixture of two soluble proteins, phosphoglycerate kinase and isocitrate dehydrogenase. In the detergent phase of the system at pH 9.0 in the presence of SDS the remainder of band 9 was identified as cytochrome BCl complex subunit A. All four soluble proteins were identified at the corresponding positions in the polymer phase of the system at pH 9.0 in the presence of SDS. This is an example of the principle that removal of soluble proteins from a complex protein mixture allows detection of a membrane protein. ADP/ATP translocator with six transmembrane α-helixes [47], voltage-dependent anion-selective channel (VDAC) with 16 transmembrane β-strands [48], ADP-ribosylation factor binding protein and ykl215cp, both with one predicted transmembrane domain according to the comprehensive yeast genome database (CYGD, http:/www.mips.gsf.de/proj/yeast/CYGD/db/index.html) (Fig. 3b, Table 1) are all examples of membrane proteins partitioning to the detergent phase regardless of pH or addition of SDS.

3.2. Improved identification by MALDI-TOF-MS of membrane proteins enriched in detergent phase separated by 1-DE

By making a closer investigation of the detergent phase from the DDM–PEG system at pH 9.0 in the presence of SDS (pre-fractionated sample), we next wanted to see

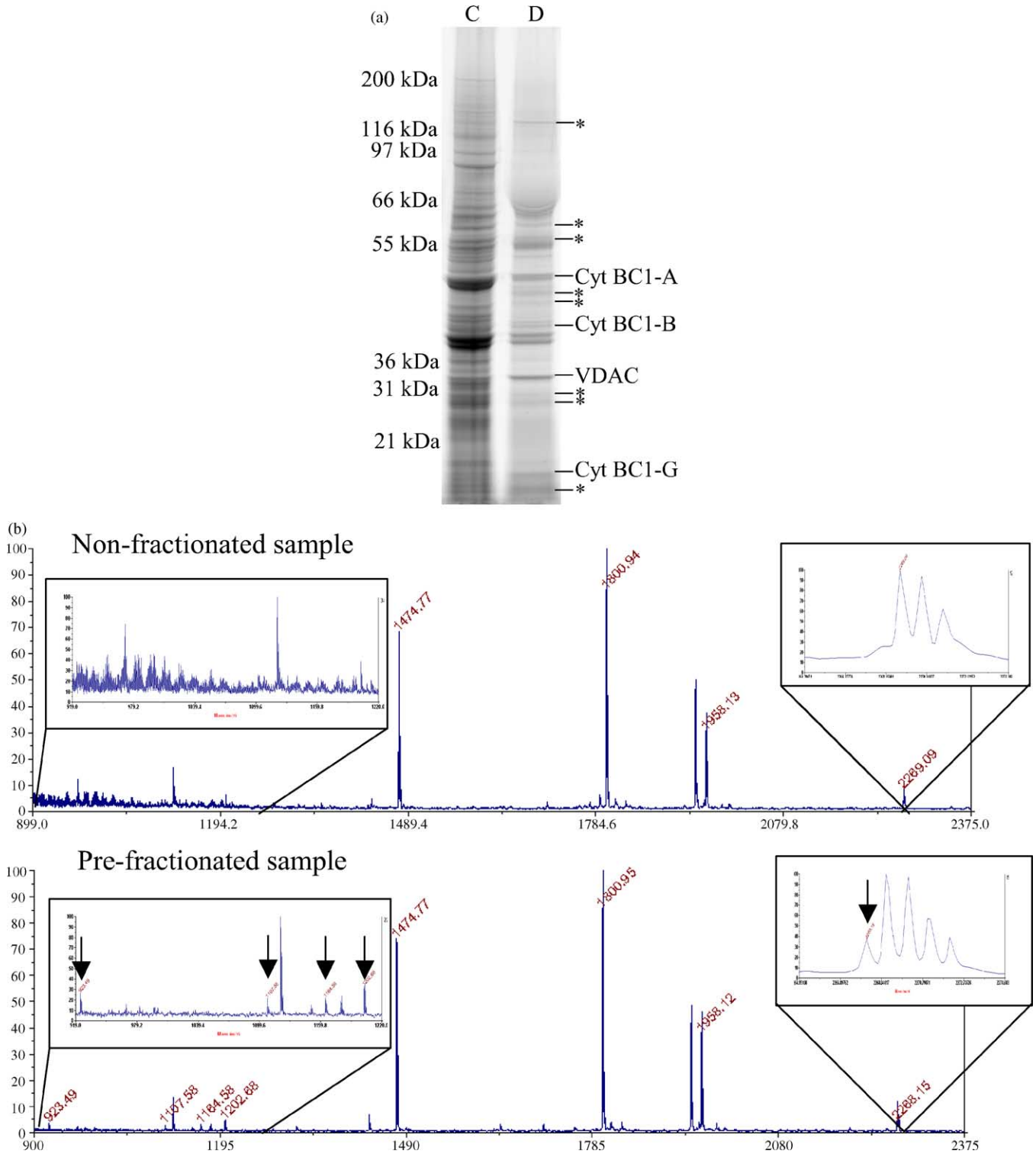

Fig. 4. Pre-fractionation in detergent–polymer two-phase system leads to facilitated detection of membrane proteins quantitatively and qualitatively. (a) Proteins enriched in the detergent phase of a DDM–PEG system at pH 9.0 in the presence of SDS. Proteins enriched in the detergent phase and the corresponding region in the control were analysed with MALDI-TOF-MS (Table 2). (C) Control (non-fractionated mitochondria). (D) Detergent phase (system at pH 9.0, 3 mM SDS). (*) No identification possible potentially indicating that the trypsin digest resulted peptides of high mass or hydrophobicity originating from membrane proteins. (b) MALDI-MS spectra of the membrane protein VDAC enriched in the detergent phase. Upper panel: non-fractionated mitochondria, lower panel: pre-fractionated sample (detergent phase from two-phase system at pH 9.0, 3 mM SDS). Highlighted regions show increased resolution of peptides. Arrows show five peptides exclusively found in the pre-fractionated sample.

Table 2
Enriched proteins with improved resolution and more reliable identification in the detergent phase after pre-fractionation in a DDM–PEG two-phase system at pH 9.0, in the presence of SDS, followed by 1-DE (Fig. 4) identified with MALDI-TOF-MS

Protein	SwissProt ID	Seq. cov. C (%)	Seq. Cov. D (%)	*Z*-value C	*Z*-value D
VDAC	P04840	17	38	0.48	**2.26**
Cyt BC1-A	P07256	ND	20	ND	**1.80**
Cyt BC1-B	P07257	31	36	**2.35**	**2.35**
Cyt BC1-G	P00128	ND	39	ND	**1.69**

C: control (non-fractionated mitochondria); D: detergent phase of system pH 9.0 + SDS; Seq. cov.: sequence coverage; ND: not detectable, positive identifications (*Z*-value > 1.65) in bold. System composition: 4% (w/w) PEG, 15% (w/w) DDM and 10 mM Tris–HCl, pH 9.0, 3 mM SDS; temperature 4 °C.

if more membrane proteins could be detected compared to non-fractionated sample (solubilised mitochondria). Resolution was indeed improved in the pre-fractionated sample compared to the non-fractionated sample (Fig. 4a, Table 2). For example, VDAC appeared distinctly in the pre-fractionated sample, whereas in the non-fractionated sample it was difficult to separate this protein from other bands.

Improved resolution is also evident from the MALDI-MS spectra (Fig. 4b) of the VDAC band from the pre-fractionated detergent phase compared to the control (non-fractionated sample). Whereas only three peptides were detected in the non-fractionated mitochondria (Fig. 4b, upper panel), eight peptides originating from VDAC were detected in the pre-fractionated sample (Fig. 4b, lower panel). In the pre-fractionated sample a decrease in background made it possible to find four additional VDAC peptides in the highlighted region M_r 900–1200 (Fig. 4b, lower panel) that were not found in this region in the non-fractionated sample. The highlighted peptide 2268.09 (Fig. 4b) was found in the pre-fractionated sample, while in the non-fractionated sample only a mass of 2269.15 (Fig. 4b, lower panel) could be detected. This is probably due to a peptide overlap from a contaminating peptide with a mass of 2269.15. Identification of VDAC from the pre-fractionated sample resulted in a *Z*-value of 2.26 and 38% sequence coverage, compared to a *Z*-value of only 0.48 and only 17% sequence coverage in the non-fractionated sample (Table 2). The cytochrome BC1 complex subunit G was detected with a *Z*-value of 1.69 and 39% sequence coverage (Table 2) from four identified peptides, in the pre-fractionated sample, whereas the peptide extract from the corresponding band in the non-fractionated sample did not allow identification of any of these proteins.

In summary, pre-fractionation with the DDM–PEG two-phase system led to improved membrane protein detection not only quantitatively but also qualitatively (Fig. 4), as reflected by better *Z*-values and higher sequence coverage (Table 2).

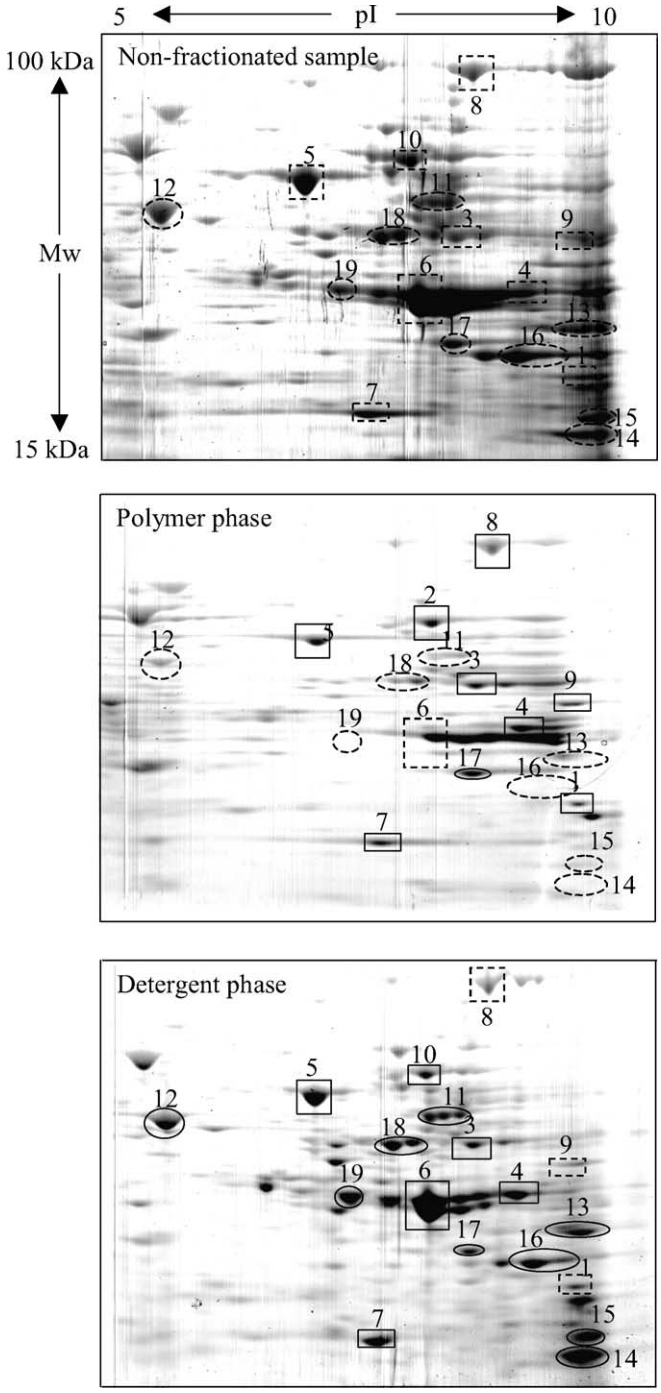

Fig. 5. 2-DE gel analysis of non-fractionated isolated yeast mitochondria and of isolated phases after pre-fractionation in a Triton X-114–PEG system. Proteins were identified using MALDI-TOF-MS and ESI-QTOF-MS–MS. Identified proteins are numbered and listed in Table 3. Circled protein spots were identified as membrane proteins and boxes denote soluble proteins. Dotted circles and boxes indicate corresponding positions of identified proteins on all gels. The two-phase system was mixed to obtain final concentrations of 11% (w/w) Triton X-114 and 4% (w/w) PEG 40 000, 10 mM Tris–HCl pH 9.0, 100 mM $NaClO_4$.

3.3. Membrane proteins enriched in detergent phase separated by conventional 2-DE

The possibility to enrich membrane proteins prior to 2-DE analysis for proteomics by pre-fractionation should be of interest to improve resolution and allow larger sample loads of these proteins. This would increase detection limits compared to the initial sample. The enrichment of membrane proteins in the detergent phase was therefore also evaluated by 2-DE. The detergent–polymer system Triton X-114–PEG was used at pH 9.0 and to enhance removal of soluble proteins $NaClO_4$ was added to the systems. The results (Fig. 5, Table 3) again showed that proteins observed in the detergent phase but not in the polymer phase were membrane proteins.

In the polymer phase, spot 2 was identified as the water-soluble protein acetyl CoA hydrolase and spot 10 in the detergent phase was identified as glycerol-3-phosphate dehydrogenase with one predicted transmembrane region in CYGD. These proteins have the same isoelectric points and molecular weights. This is an example of improved resolution of membrane proteins in 2-DE after pre-fractionation in a detergent–polymer two-phase system. This can be compared to earlier results by Wissing et al. [20] where a membrane protein and a soluble protein with the same isoelectric points and molecular masses were separated by cloud point extraction with Triton X-114. In the detergent–polymer system there is no need to raise the temperature in the pre-fractionation step. Spot 17 was identified to be NADH-cytochrome B5 reductase, a protein found in the mitochondria in two different isoforms, one membrane bound and one soluble. An explanation for the presence of spot 17 both in the polymer phase and the detergent phase could be that the soluble isoform of the protein was in excess and partitioned to the polymer phase and the membrane bound isoform was partitioned to the detergent phase.

By MALDI-TOF-MS and ESI-QTOF-MS–MS analyses of a number of protein spots (Fig. 5), we succeeded in identifying 10 membrane proteins, only three with transmembrane domains, enriched in the detergent phase. The corresponding proteins were not, or only to a small extent, detected in the polymer phase (Table 3). The fact that 5 subunits representing the water-soluble membrane associated region (F1) of the ATP-synthase were found enriched in the detergent phase suggests that this protein complex stays intact in the two-phase system.

3.4. Membrane proteins enriched in detergent phase further fractionated by ion-exchange chromatography followed by 1-DE

Isoelectrical focusing of hydrophobic proteins, especially membrane proteins, in gel electrophoresis often lead to aggregation of the proteins in the gel-strip causing under-representation of these proteins in the second dimension of a 2-DE gel. To avoid the losses of membrane proteins in an isoelectric focusing step, the membrane proteins enriched in the detergent phase from the DDM–PEG system were further fractionated in two dimensions by ion-exchange chromatography followed by 1-DE on the eluted fractions (Fig. 6). After pre-fractionation in DDM–PEG two-phase system, ion-exchange chromatography and 1-DE, 10 membrane proteins with one or more transmembrane domain were identified with MALDI-TOF-MS (Table 4). This

Table 3
Proteins identified from 2-DE (Fig. 5) with MALDI-TOF-MS and ESI-QTOF-MS–MS after pre-fractionation in a Triton X-114–PEG two-phase system

Number	Protein name	SwissProt ID	M_r ($\times 10^{-3}$)	p*I*	TMD
1	Phosphoglycerate mutase	P00950	27.4	8.9	
2	Acetyl CoA hydrolase	P32316	58.9	6.3	
3	Phosphoglycerate kinase	P00560	44.7	7.1	
4	Isocitrate dehydrogenase	P28834	39.3	9.0	
5	Aldehyde dehydrogenase	P00360	57.0	6.3	
6	Branched chain amino acid biosynthesis, Ilv5	P06168	44.5	9.2	
7	Superoxid dismutase	P00445	22.7	5.9	
8	Aconitase	P19414	85.3	8.2	
9	Mitochondrial respiratory function protein 1	P38071	40.9	8.9	
10	Glycerol-3-phosphate dehydrogenase	P32191	72.4	8.0	1
11	ATP-synthase, subunit α	P07251	58.6	9.0	MA
12	ATP-synthase, subunit β	P00830	54.9	5.7	MA
13	ATP-synthase, subunit γ	P38077	34.4	9.4	MA
14	ATP-synthase, subunit D	P30902	19.8	8.9	MA
15	ATP-synthase, oligomycin sensitivity conferral protein	P09457	22.8	9.6	MA
16	Voltage-dependent anion-selective channel protein	P04840	30.6	6.8	16
17	NADH-cytochrome B5 reductase	P36060	34.2/32.0	8.7	1/0[a]
18	Cytochrome BC1 complex subunit A (Cyt BC1-A)	P07256	50.2	6.8	MA
19	Cytochrome BC1 complex subunit B (Cyt BC1-B)	P07257	40.5	7.7	MA

[a] The protein occurs in two isoforms, one membrane bound and one water-soluble. The two-phase system composition was: 11% (w/w) Triton X-114 and 4% (w/w) PEG 40 000, 10 mM Tris–HCl, pH 9.0, 100 mM $NaClO_4$.

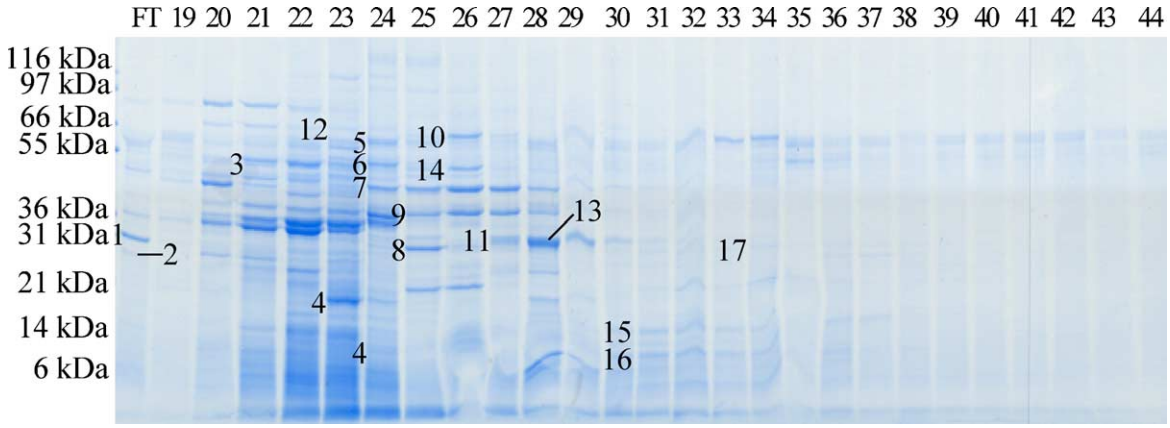

Fig. 6. Two-dimensional separation of membrane proteins enriched in the detergent phase of a DDM–PEG two-phase system at pH 9.0 and in the presence of SDS, using anion-exchange chromatography in combination with 1-DE of the collected fractions. Isolated yeast mitochondria were pre-fractionated in dodecyl maltoside (DDM)–poly(ethylene glycol) (PEG) two-phase system (total weight 3 g) at pH 9.0, 3 mM SDS. The detergent phase was diluted to 7.0 ml with 20 mM Tris–HCl, pH 8.0, 0.25% DDM (buffer A) and applied to a Resource Q1 ml anion-exchange column connected to an ÄKTA explorer system. Elution: 0–50% 20 mM Tris–HCl, pH 8.0, 0.25% DDM, 2.0 M NaCl over 40 column volumes, followed by a step elution with 100% over 5 column volumes. Eluted protein fractions were detected at 280 nm and collected in 1 ml fractions analysed by subsequent SDS-PAGE (fraction numbers on top of the lanes). The 20 membrane proteins identified by MALDI-TOF-MS are numbered on the gel and listed in Table 4.

Table 4
Identified membrane proteins after pre-fractionation in a DDM–PEG two-phase system followed by a two-dimensional separation using anion-exchange chromatography and SDS-PAGE (Fig. 6)

Number	Protein name	SwissProt ID	p*I*	TMD
1	ADP/ATP translocator	P18239	9.9	6
2	Voltage-dependent anion-selective channel (VDAC)	P04840	9.9	16
3	NADH-ubiquinone oxidoreductase	P32340	9.4	1
4	Mitochondrial outer membrane protein	P16547	8.5	1
5	Succinate dehydrogenase, flavoprotein subunit	Q00711	7.2	MA
	L-Galactono-γ-lactone oxidase	P54783	6.4	1
6	Glycerol 3-phosphate dehydrogenase	P32191	8.0	1
7	Cytochrome BC1 complex subunit A (Cyt BCl-A)	P07256	6.8	MA
8	NADH-cytochrome B5 reductase	P36060	8.7	1
9	Cytochrome BC1 complex subunit B (Cyt BC1-B)	P07257	7.7	MA
10	Carnitine O-acetyltransferase	P32796	8.5	MA
11	Prohibitin	P40961	7.9	0.5[a]
12	ATP synthase, β-subunit	P00830	5.5	MA
13	Cytochrome *c* 1	P07143	8.5	0.5[a]
14	NADPH-cytochrome P450 reductase	P16603	5.0	0.5[a]
	ATPase, VO-subunit	P32563	5.3	7
15	Succinate dehydrogenase, cytochrome *b* subunit	P33421	10.2	3
	Cytochrome *c* oxidase, subunit IV	P04037	6.4	MA
16	Cytochrome *c* oxidase, subunit VI	P00427	5.8	MA
17	Cytochrome *c* oxidase, subunit II	P00410	4.7	2

Two-phase system composition: 4% (w/w) PEG, 15% (w/w) DDM, 10 mM Tris–HCl, pH 9.0, 3 mM SDS. Chromatography conditions: resource Q 1 ml anion-exchange column. Buffer (A) 20 mM Tris–HCl, pH 8.0, 0.25% DDM. Elution: 0–50% buffer (B) 20 mM Tris–HC1, pH 8.0, 0.25% DDM, 2.0 M NaCl, 40 column volumes, 100% buffer B, 5 column volumes.

[a] 0.5: Membrane anchored protein.

should be compared with four transmembrane proteins when no further fractionation was made prior to 1-DE (Table 1), and only three transmembrane proteins from 2-DE (Table 3).

Thus, reducing sample complexity by removing abundant soluble proteins using pre-fractionation with two-phase partitioning followed by ion-exchange chromatography and 1-DE offers a potential for more efficient studies of membrane proteins. This method allows exclusion of the isoelectric focusing step in 2-DE, thus, circumventing the problem of aggregation of hydrophobic proteins in the electrophoresis gel matrix, which is beneficial for yield and resolution of hydrophobic proteins.

4. Concluding remarks

Detergent/polymer aqueous two-phase system partitioning provides a novel, fast and mild method for

pre-fractionation of complex protein mixtures. Membrane proteins are enriched in a detergent phase and separated from abundant water-soluble proteins and insoluble materials that are removed to the polymer phase at low temperatures. The described method allows enrichment of membrane proteins in a small volume, and at the same time allows high loading of complex biological material such as solubilised mitochondria where only a fraction of the proteins are membrane proteins. Thus, this makes detergent–polymer aqueous two-phase systems a suitable method for lowering the complexity of protein mixtures before the use of high-resolution separation techniques, such as ion-exchange chromatography and one- or two-dimensional gel electrophoresis. By using a detergent–polymer two-phase system in combination with ion-exchange chromatography and SDS-PAGE an improved resolution can be reached for membrane proteins with an increased resolution of low abundance peptides in MS spectra because of the removal of peptides originating from contaminating proteins. Thus, higher sequence coverage and more reliable protein identifications can be achieved. The detergent–polymer two-phase systems offer a promising alternative to currently used pre-fractionation methods for identification and characterization of the many membrane proteins of different proteomes.

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