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Rapid prefractionation of complex protein lysates with centrifugal membrane adsorber units improves the resolving power of 2D-PAGE-based proteome analysis

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Abstract

Background: Two-dimensional gel electrophoresis (2D-PAGE) has proven over the years to be a reliable and efficient method for separation of hundreds of proteins based on charge and mass. Nevertheless, the complexity of even the simplest proteomes limits the resolving power of 2D-PAGE. This limitation can be partially alleviated by sample prefractionation using a variety of techniques.

Results: Here, we have used Vivapure Ion Exchange centrifugal adsorber units to rapidly prefractionate total fission yeast protein lysate based on protein charge. Three fractions were prepared by stepwise elution with increasing sodium chloride concentrations. Each of the fractions, as well as the total lysate, were analyzed by 2D-PAGE. This simple prefractionation procedure considerably increased the resolving power of 2D-PAGE. Whereas 308 spots could be detected by analysing total protein lysate, 910 spots were observed upon prefractionation. Thorough gel image analysis demonstrated that prefractionation visualizes an additional set of 458 unique fission yeast proteins not detected in whole cell lysate.

Conclusions: Prefractionation with Vivapure Q spin columns proved to be a simple, fast, reproducible, and cost-effective means of increasing the resolving power of 2D-PAGE using standard laboratory equipment.

Background

Despite some limitations, 2-dimensional gel electrophoresis (2D-PAGE) [1] coupled to mass spectrometric protein identification remains one of the most reliable and reproducible means of proteome analysis. Due to the wide dynamic range of individual proteins in cells, which presumably varies over five to six orders of magnitude [2], a key requirement for a comprehensive proteome analysis is to reduce sample complexity to a level that permits access to low abundance proteins. Whereas the resolving

power of 2D-PAGE is remarkable, biochemical prefractionation will further enhance resolution enabling a deeper view into complex proteomes (reviewed in [3]).

Prefractionation can be achieved by a number of techniques such as differential protein extraction, purification of cell organelles or protein complexes, preparative isoelectric focusing (IEF), or chromatographic techniques [4]. Many of these procedures are time consuming, difficult to reproduce and scale up, result in sample loss, and often require expensive instrumentation such as liquid chromatography systems.

Here, we have tested Vivapure Ion Exchange Spin Columns employing a membrane adsorber technology as the chromatography matrix to fractionate proteins based on differences in charge. Unlike traditional chromatography resins, membrane adsorbers make use of convective transport to bring proteins to the ion exchange surface in microcentrifuge format. Hence, the binding, washing, and elution steps are performed rapidly in standard laboratory equipment. Moreover, the use of low binding materials together with centrifugal elution ensures highly efficient sample recovery.

While this method should have application to a broad range of cell types, we demonstrate here its utility for increasing the resolving power of 2D-PAGE-based proteome analysis of the fission yeast *Schizosaccharomyces pombe*.

Results and discussion

Total *S. pombe* cell lysate was prepared by bead lysis as described in the Methods section. An aliquot of this lysate was precipitated with trichloroacetic acid (TCA), followed by resuspension of the protein pellet in IEF buffer. 0.8 mg of protein was separated by IEF on nonlinear pH 3–10 immobilized gradient strips and second dimension SDS gel electrophoresis. Gels were stained with SYPRO Ruby, and stained protein spots were detected by fluorescence scanning. Image analysis revealed that this technique detected 308 unique protein spots in total *S. pombe* cell lysate (Fig. 1A). In contrast, the theoretical proteome of this organism comprises 4395 proteins within a pI range between 3.0 and 10.0 (Fig. 1B).

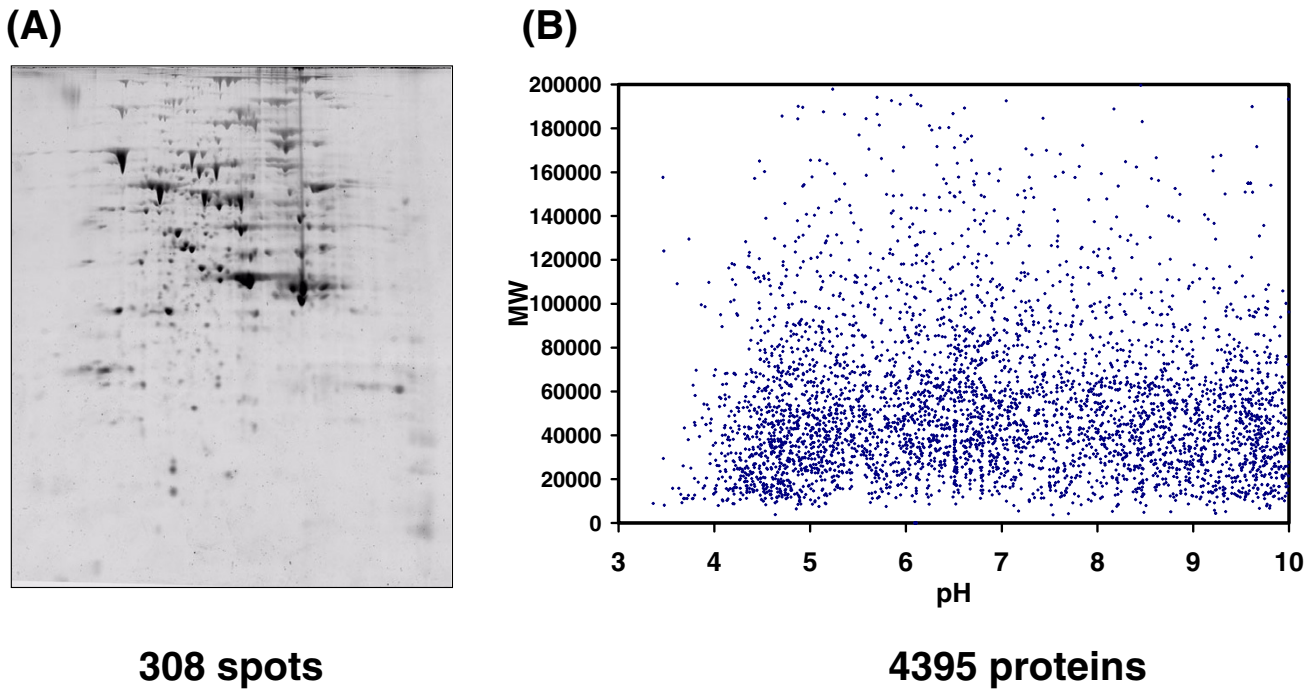


Figure 1
Practical and theoretical 2D-PAGE analysis of total *S. pombe* cell lysate. (A) 0.8 mg total *S. pombe* protein lysate was separated by 2D-PAGE as described in Methods. The gel was stained with SYPRO Ruby and analyzed with the Phoretix 2D imaging software. **(B)** All proteins in the Sanger Centre *S. pombe* genome database with pI values between 3.0 and 10.0 possessing a molecular weight below 200 kDa were blotted in the diagram to indicate a virtual whole cell 2D-PAGE.

To increase our penetration of the fission yeast proteome, we sought a simple and reproducible method to prefractionate cell lysate using Vivapure anion exchange spin columns. 4 mg total cell protein was applied to the column in a volume of 400 ul and eluted with increasing concentrations of sodium chloride in increments of 50 mM. Fig. 2 summarizes the binding and elution characteristics of the spin column. Binding efficiency to the membrane adsorber was ~55% of the total protein loaded, and ~94% of bound protein was recovered at the end of the step gradient at 1 M NaCl (Fig. 2A,2B). 38% of the loaded protein eluted between 50 mM and 500 mM NaCl. Gel analysis of individual fraction revealed a different pattern of proteins in the fractions eluted with 225 mM, 300 mM, and 500 mM NaCl (Fig. 2C).

Based on these findings, we devised the 2D-PAGE compatible prefractionation scheme outlined in Fig. 3A. In brief, total cell lysate was precleared by ultracentrifugation, loaded on a Vivapure anion exchange column, followed by separation into three fractions with step gradients of 225 mM, 300 mM, and 500 mM NaCl. Samples were then precipitated with TCA/acetone, resuspended in IEF buffer, and analyzed by 2D-PAGE.

Fig. 3B shows the elution profiles of two independent samples. The nearly complete overlap of the two curves indicated that the prefractionation and step elution was highly reproducible. In addition, the three fractions contained a similar amount of protein (~0.5 mg). The 2D-PAGE analysis showed that prefractionation enriched for many proteins that were not visible in unfractionated lysate (Fig. 3C). Whereas 308 spots were visible in the total cell lysate, 307, 302, and 283 spots were observed in fractions 1, 2, and 3, respectively (Fig. 3C and Table 1). In addition, as expected for successful fractionation by charge, there was a successive enrichment of more acidic proteins as the NaCl concentration of the elution buffer increased (Fig. 3C). Overall, the spin column prefractionation led to a similar deconvolution of sample complexity as we had previously observed with anion exchange chromatography on an automated FPLC system [4].

More detailed comparative image analysis of corresponding gel areas revealed many spots that were only detectable upon prefractionation. As an example, a zoom-in image of a section of the fraction 3 gel (elution with 500 mM NaCl) showed an array of acidic proteins undetectable in total lysate (Fig. 4A). Further quantitation showed that 156 proteins were uniquely enriched in fraction 1, 175 in fraction 2, and 127 in fraction 3, but not present in total cell lysate (Fig. 4B). Alltogether, 766 unique spots can be visualized in the three fractions.

Conclusions

Prefractionation with centrifugal membrane adsorber units was found to be a simple and reliable method to increase the resolving power of 2D-PAGE. Prefractionation led to a threefold increase in the number of features descernible by 2D-PAGE. Approximately 50% of these features are uniquely represented in the fractions, but not in total protein lysate.

Methods

Preparation of total cell lysate

A liquid culture of 50 ml fission yeast cells (*927 h-leu1-32 ura4-d18*) was grown at 30°C to an OD_{595} of 1.5. Cells were harvested by centrifugation, frozen at -80°C, and resuspended in 500 ul lysis buffer (100 mM sodium bicarbonate pH 8.8, 0.5% Triton X100, and protease inhibitors (1 mM PMSF, 10 ug/ml leupeptin, 10 ug/ml pepstatin, 15 ug/ml aprotinin)). Cells were disrupted by bead lysis in a microfuge tube containing 0.5 ml Zirconia beads (0.5 mm, Biospec Products. Inc.) and the cell debris was removed by centrifugation for 15 minutes at 4°C. The protein concentration was determined with the BioRad DC Protein Assay using BSA as a standard. 1 ml protein lysate (~10 mg/ml) was incubated with 100 ug/ml RNAseA and DNAseI for 15 minutes on ice and cleared by ultracentrifugation at 395,000 × g for 30 minutes at -20°C.

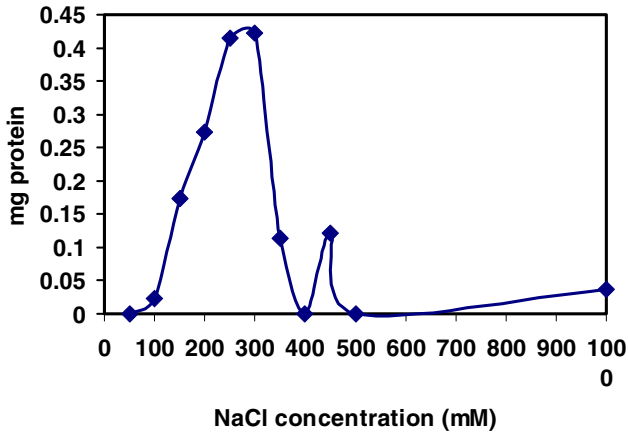
Prefractionation on membrane adsorber units

A Vivapure Q Mini spin column was equilibrated with 100 mM sodium bicarbonate buffer, pH 8.8 by loading 400 µl onto the column and spinning at 2000 × g for 5 minutes. 400 ul lysate (= 4 mg protein) was loaded onto the equilibrated spin column and spun at 2000 × g for 5 minutes. After binding, the column was washed twice with 400 ul 100 mM sodium bicarbonate, pH 8.8. Fraction 1 was eluted by loading 400 ul 100 mM sodium bicarbonate, pH 8.8 containing 225 mM NaCl onto the column and spinning at 2000 × g for 5 minutes. Subsequent elutions were performed with 400 ul 100 mM sodium bicarbonate buffer, pH 8.8, 300 mM (fraction 2) or 500 mM NaCl (fraction 3), respectively.

2D-PAGE analysis

Fractions, as well as the total lysate, were quantified and then precipitated by adding 3 volumes of chilled (-20°C) 13.3 % TCA / 0.093% 2-mercaptoethanol in acetone, followed by incubation overnight at -20°C. Samples were centrifuged at 5000 × g at -20°C and pellets were resuspended in chilled (-20°C) acetone, containing 0.07% 2-mercaptoethanol. Samples were spun again at 5000 × g at -20°C. All acetone was removed and the pellets left to dry at 30°C. The pellets were then redissolved at 30°C in CHAPS buffer (7 M urea, 2 M thiourea, 2% CHAPS, 2% ampholytes 3–10, 65 mM DTT, 0.1% bromophenol blue)

(A)



(B)

	mg	% of total protein loaded	% of bound protein
Load	**4.08**	**100.00**	
Flowthrough	**1.78**	**43.63**	
Bound before wash	**2.3**	**56.37**	
Wash	**0.62**	**15.20**	
Bound after wash	**1.69**	**41.42**	**100.00**
Eluted	**1.58**	**38.73**	**93.49**

(C)

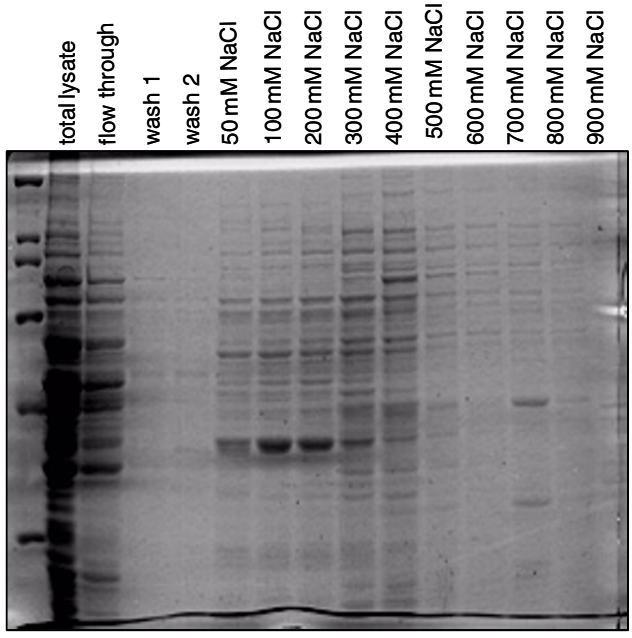


Figure 2

Sample prefractionation with Vivapure Q Mini spin columns (A) 4 mg total fission yeast protein was loaded onto a Vivapure Q mini spin column, washed, and eluted with a NaCl step gradient from 50 mM to 1 M. The elution profile is shown. **(B)** Summary of the binding and elution characteristics of Vivapure Q Mini columns. **(C)** Analysis of fractions by 1D PAGE. The flowthrough as well as low and high salt fractions showed different protein patterns.

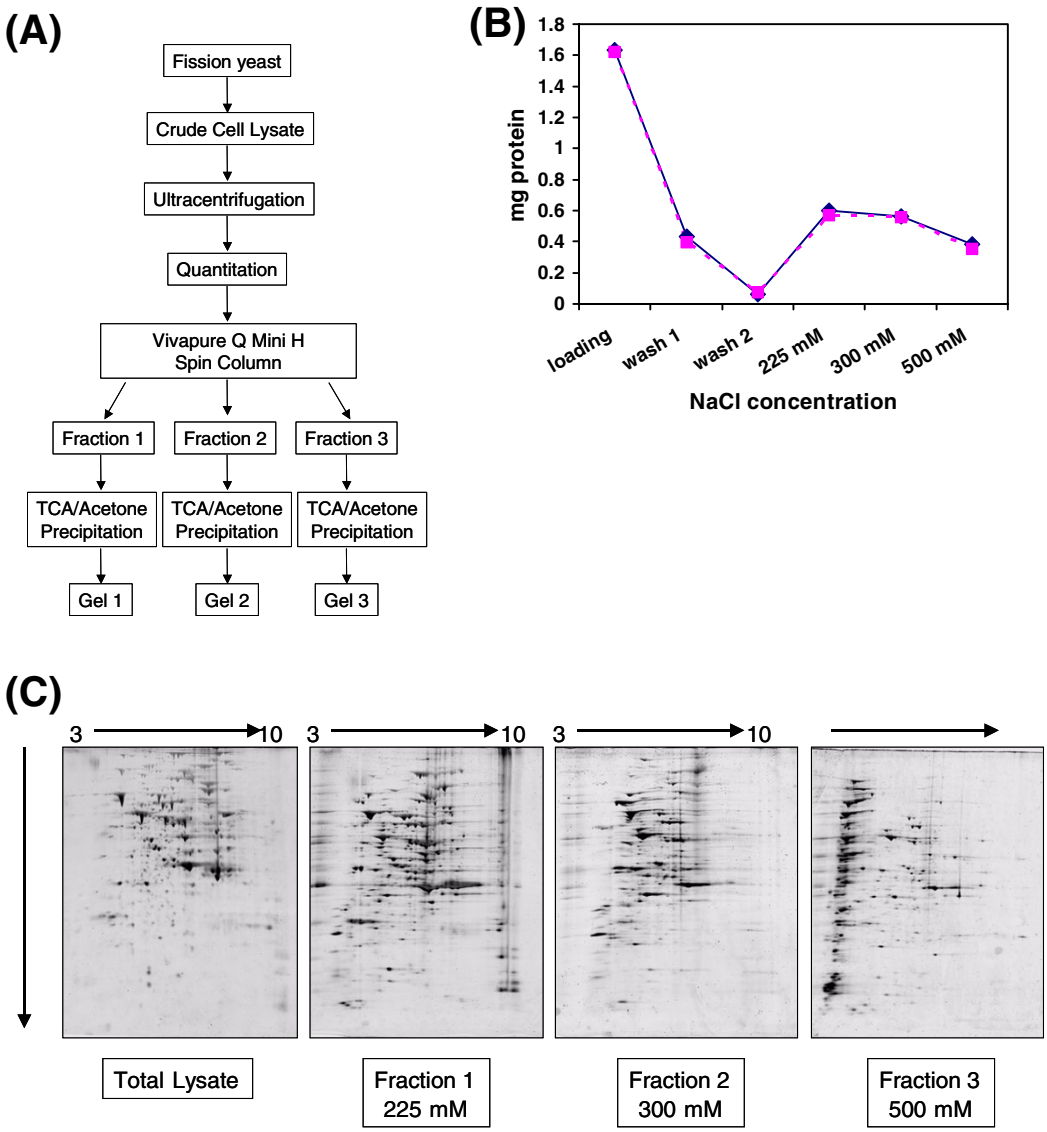
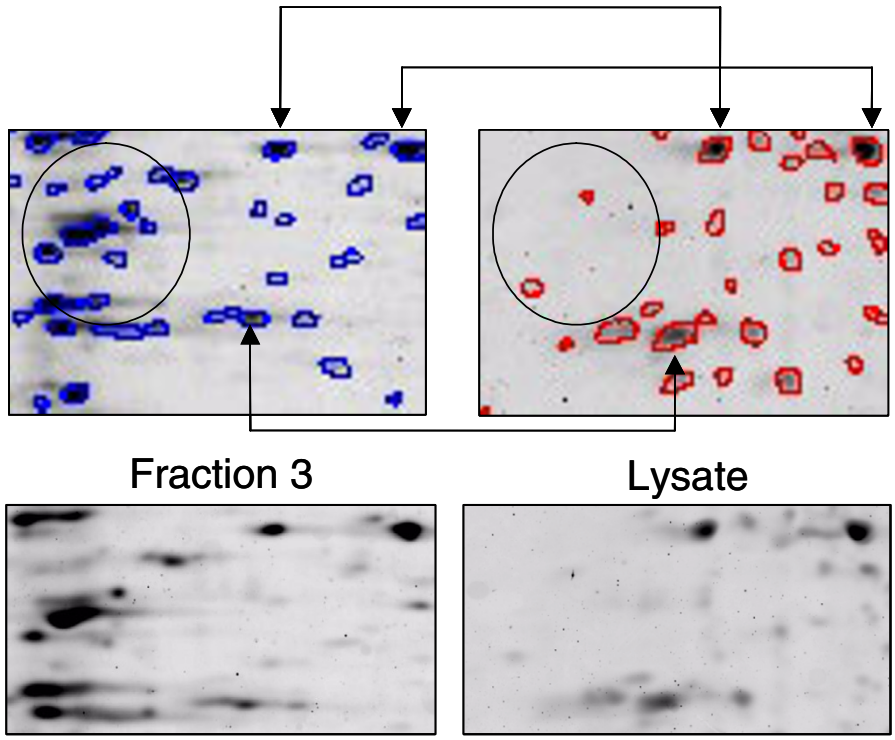


Figure 3
2D-PAGE analysis of prefractionated *S. pombe* cell lysate (A) Workflow for fractionation using Vivapure Q spin columns by applying increasing NaCl concentrations for elution. See Methods for details. **(B)** Reproducibility of the prefractionation. Two independently prepared whole cell lysates were prefractionated on Vivapure Q Mini columns and eluted into three fractions with the indicated concentrations of NaCl. The protein contents of the eluates are plotted. **(C)** 2D-PAGE of the three fractions compared to whole cell lysate.

(A)



(B)

	Number of Spots	Unique Spots
Total Lysate	**308**	**308**
Fraction 1	**307**	**156**
Fraction 2	**302**	**175**
Fraction 3	**283**	**127**
Total Number of Spots in Fractions	**910**	**458**
Total Number of Unique Spots		**766**

Figure 4
Detailed analysis of 2D-PAGE results (A) Corresponding sections of the total lysate and fraction 3 gels are shown and spots only detected upon prefractionation are indicated. **(B)** Summary of the quantitation of the 2D-PAGE results. Image analysis was performed to identify protein spots that are only detected in a single fraction, but not in the total cell lysate.

to a concentration of 2.5 mg/ml. 400 ul (1 mg) of this was loaded onto an 18 cm immobilized pH 3–10 nonlinear gradient strip (Amersham) and passively rehydrated for 16 hours. The strips were then focused to 100,000 Vh (Genomic Solutions Investigator), equilibrated in 10 ml equilibration buffer I (6 M urea, 375 mM Tris/HCl pH 7.4, 2% SDS, 2% glycerol, 2% DTT), followed by 10 ml equilibration buffer II (6 M urea, 375 mM Tris/HCl pH 7.4, 2% SDS, 2% glycerol, 2% iodoacetamide), and applied to an 8–18% gradient Duracryl SDS PAGE gel (Genomic Solutions). The gels were stained with SYPRO Ruby and imaged on a BioRad Molecular Imager FX Pro Plus (532 nm excitation, 555 nm emission filter, 1064 nm excitation filter).

Image analysis

The images were analyzed using Phoretix 2D software from Nonlinear Dynamics. Spots were detected using automatic spot detection and the background was subtracted using the mode of non-spot method. The images were also subjected to spot filtering in order to remove spots occurring as a result of residual dye crystals. The three fractions were matched against the total lysate by placing thirty seed matches from which automatic matches were then made. The three fractions were also matched against one another by the same method to determine the number of unique spots in each fraction.

List of abbreviations

2D-PAGE; two-dimensional polyacrylamide gel electrophoresis

IEF; isoelectric focusing

SDS; sodium dodecyl sulfate

TCA; trichloroacetic acid

Competing interests

MKD received $500 from Vivascience AG toward travel costs to the 2003 HUPO meeting in Montreal. No other competing interests.

Authors' contributions

MKD performed all the experiments shown in Fig. 1A,2, and 3. MWS contributed the data shown in Fig. 1B. CN, AK, and NKP contributed to the development and optimization of the Vivapure spin column protocols. DAW, RZ, and DH conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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