

Efficient Protein Fractionation and Identification with the Agilent 3100 OFFGEL Fractionator

Application Note

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Abstract

The sample fractionation steps done prior to mass detection are critically important for the comprehensive analysis of complex protein mixtures. This Application Note illustrates the potential of OFFGEL electrophoresis with the Agilent 3100 Fractionator for the fractionation of proteins. An *E. coli* lysate was separated into 24 fractions, proteins were digested with Trypsin and peptides were identified by reversed phase liquid chromatography on a microfluidic device with mass spectrometric detection. The accuracy and reproducibility of the OFFGEL fractionation was shown by one and two dimensional gel electrophoresis respectively. Compared to an experiment without OFFGEL fractionation, a more than threefold increase in the number of identified proteins and a ninefold increase in the number of identified peptides were observed.

Introduction

The success of proteome analysis projects is highly dependent on the quality of the sample fractionation steps employed prior to analysis by mass spectroscopy (MS). A widely used technique for the analysis of complex protein samples is two-dimensional gel electrophoresis (2DE). This method allows high resolution separations, but is technically demanding and difficult to automate. Furthermore, large scale analysis of proteoms is challenging because protein spots have to be visualized by staining, individually excised, digested and extracted from the gel prior to analysis by MS, which is a tedious and time-consuming process. An alternative to 2DE is offered by the OFFGEL technology^{1,2}. This method takes advantage of the impressive separation power of immobilized pH gradient based isoelectric focusing (IPG IEF) but recovers sample in the liquid phase. After fractionation, proteins can be directly digested and analyzed by LC/MS. No gel staining and no sample extraction from the gel are required. Furthermore, separations with the Agilent 3100 OFFGEL fractionator are easy to perform and have the potential for automation.

Experimental

Sample preparation

Lyophilized *E. coli* strain B cells (ATCC 11303; Sigma, Taufkirchen, Germany) were resuspended in lysis buffer (7M Urea, 2M Thiourea, 1 % DTT) and disrupted with a BeadBeater™ (Biospec Products, Bartlesville, OK, USA). Cell debris was removed by centrifugation at 25000 xg and 4 °C for 30 min. The protein concentration of the cell lysate was determined with the Coomassie Plus Assay Reagent (Pierce, Rockford, IL, USA).

OFFGEL electrophoresis

For pI based protein separation, the Agilent 3100 OFFGEL Fractionator with a 12- or a 24-well setup (3100 OFFGEL Low Res Kit, pH 3-10, Agilent part number 5188-6425, or High Res Kit, pH 4-7, Agilent part number 5188-6426) was used according to the protocol. The *E. coli* cell lysate was diluted with Protein OFFGEL Solution and 0.15 mL diluted sample was loaded per well. The default methods 'OG24PR00' and 'OG12PR00' were used: limits 'Focusing' phase: 64 kVh and 20 kVh for OG24PR00 and OG12PR00 respectively, 8000 V, 50 µA, 200 mW, and 100 h; limits 'Hold' phase: 500 V, 20 µA and 50 mW. The temperature was set to 20 °C.

Tryptic digest

5 µL 1 M ammonium bicarbonate and 10 µL 1 M iodoacetamid (freshly dissolved in 50 mM ammonium bicarbonate) were added to every fraction. After 30 min incubation in the dark, non-reacted iodoacetamide was

quenched by the addition of 5 µL 1 M DTT. Fractions were desalted by gel filtration with 0.5 mL Zeba Desalt Spin Columns (Pierce, Rockford, IL, USA). After addition of 5 µL 1 M ammonium bicarbonate, Trypsin (freshly dissolved in 50 mM ammonium bicarbonate; Pierce, Rockford, IL, USA) was added at 1:30 enzyme:substrate and fractions were incubated overnight at 37 °C. The digest was stopped with 20 µL 1 % formic acid and peptides were lyophilized, resuspended in 0.1 mL 0.1 % formic acid, lyophilized again and stored at -20 °C. As control for an analysis without OFFGEL fractionation, 0.1 mg *E. coli* lysate was diluted with Protein OFFGEL Stock Solution and digested in the same way as the OFFGEL fractions.

One and two dimensional electrophoresis

For 2DE, 50 µL aliquots of OFFGEL fractions were diluted with Protein OFFGEL Solution and loaded by overnight rehydration at room temperature on 7 cm IPG gel strips pH 4-7. Samples were focused for 8 kVh with an upper current limit of 50 µA at 20 °C. For 2DE analysis of the unfractionated control, 0.25 mg *E. coli* cell lysate was diluted with Protein OFFGEL Stock Solution and loaded by overnight rehydration at room temperature on 13 cm IPG gel strips pH 4-7. Isoelectric focusing was done for 16 kVh with an upper current limit of 50 µA at 20 °C. Prior to running the second dimension, 3 cm gel were removed on both ends of the IPG strip.

Strip equilibration and SDS-PAGE on precast 4-12 % BisTris minigels were done according to the supplier's instructions (Invitrogen, Carlsbad, CA, USA). Gels were stained with PhastGel Blue R-350 (GE Healthcare, Freiburg, Germany). As molecular weight standard, the Mark 12™ Unstained Standard was used (Invitrogen, Carlsbad, CA, USA).

HPLC-Chip/MS analysis

For analysis by LC/MS, lyophilized peptides were resuspended in 100 μL 1 % formic acid. A 1-2 μL aliquot of each fraction was injected onto an LC/MS system consisting of an 1100 Series liquid chromatograph, HPLC-Chip Cube MS interface, and 1100 Series LC/MSD Trap XCT Ultra ion trap mass spectrometer (all Agilent Technologies). The system was equipped with an HPLC-Chip (Agilent Technologies) that incorporated a 40 nL enrichment column and a 150 mm x 75 μm analytical column packed with ZORBAX 300SB-C18 5 μm particles. Peptides were loaded onto the enrichment column with 97 % solvent A (water with 0.1 % formic acid) and 3 % B (acetonitrile with 0.1 % formic acid) at 4 $\mu\text{L}/\text{min}$. They were then eluted with a gradient from 3 % B to 40 % B in 60 min, followed by a steep gradient to 80 % B in 1 min at a flow rate of 0.3 $\mu\text{L}/\text{min}$. The total run-time of the used gradient including column reconditioning was 65 min. The SwissProt database was searched with the restriction to *E. coli*, using the Agilent Spectrum Mill Server software. An iterative searching strategy was employed

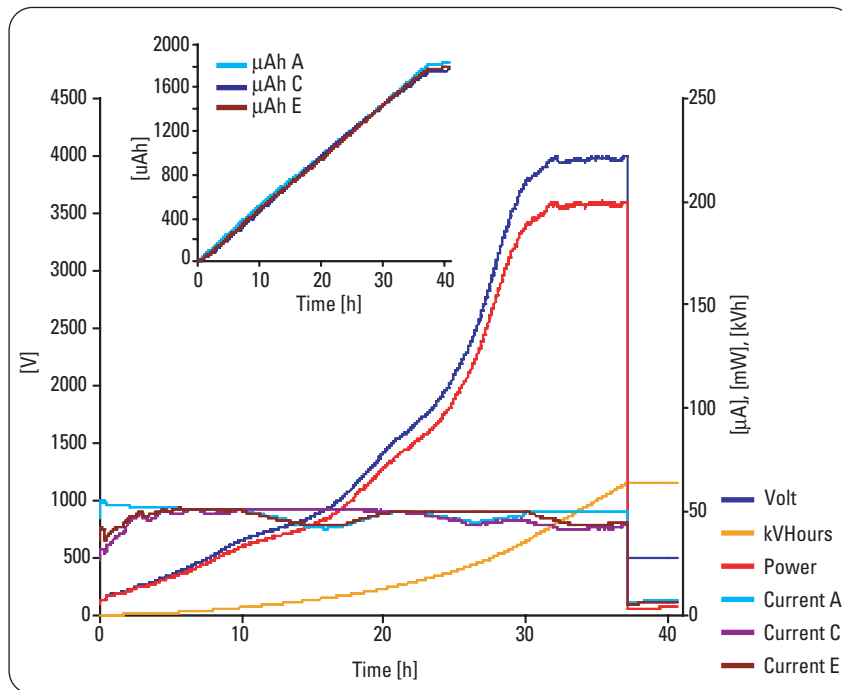


Figure 1
Current/voltage plot of three parallel OFFGEL runs in lanes A, C and E of tray 1 of the Agilent 3100 OFFGEL Fractionator. The inset shows a plot of the acquired electric charge (in μAh).

for all searches. Autovalidation flagged the high confidence identifications so that no false positives were included.

Results and discussion

Three parallel OFFGEL runs with a load of 1 mg *E. coli* protein lysate per lane were performed on a single tray of the Agilent 3100 OFFGEL Fractionator. The current/voltage profiles of the fractionation are shown in figure 1. The Agilent 3100 OFFGEL Fractionator automatically measures run parameters current and the acquired electric charge (in μAh) for every individual sample. This data is plotted online on the handheld controller and can also easily be

exported via an USB-interface and analyzed in Excel. A macro simplifying this step can be downloaded free of charge from www.agilent.com/chem/OFFGEL. During the entire focusing phase, the current was the limiting factor. The sample that was running with 50 μA changed, but the current values for all three samples were very similar during the whole run. This is also reflected by a plot of the acquired electric charge (in μAh) (insert figure 1). The acquired ampere hours are an important experimental parameter for judging the fractionation quality of individual samples. The lines for the three samples run almost parallel indicating a reproducible fractionation. After 64 kVh were reached, a hold phase automatical-

ly kept the samples at a low voltage to prevent protein diffusion before the experiment was stopped by the user. The good reproducibility of the fractionation could be demonstrated by strikingly similar SDS-PAGE results of three independent runs (figure 2). To show the high resolution of the method four neighbouring fractions (9-12) of a single OFFGEL run were analyzed by 2DE (figure 3). Proteins focused in sharp fractions in pH intervals down to 0.1 pH units, with only little overlap between neighbouring wells. As a comparison, figure 3 shows the 2D gel of the unfractionated *E. coli* lysate with the first dimension IEF prepared by the 3100 OFFGEL Fractionator.

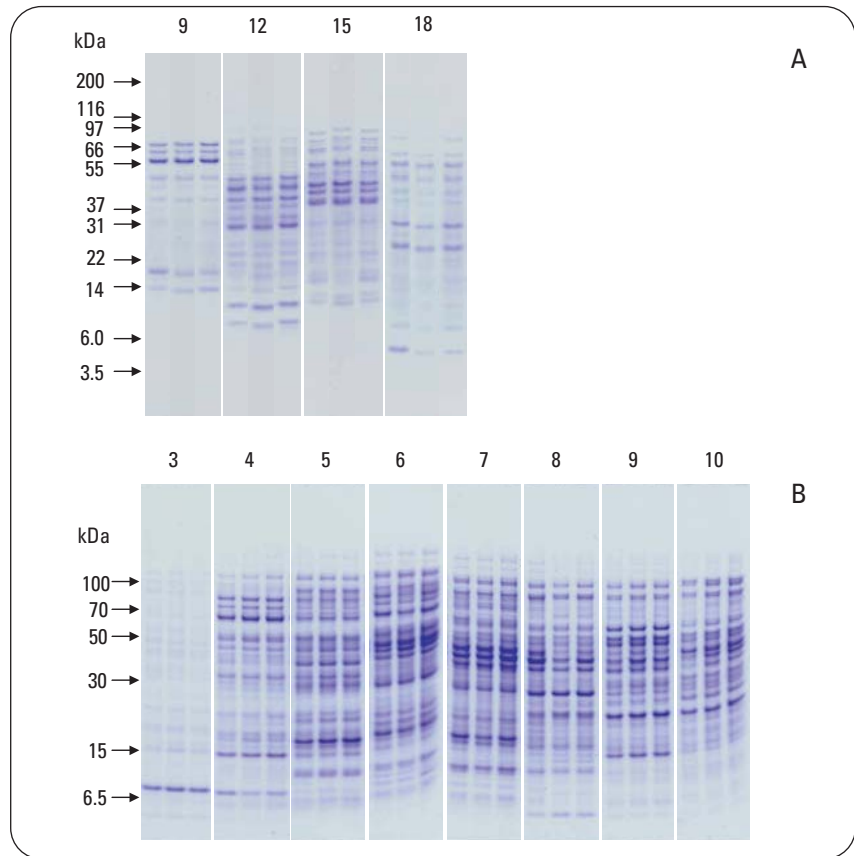


Figure 2
Reproducibility of OFFGEL electrophoresis. A) 1 mg *E. coli* lysate was fractionated into 24 (pH 4-7) fractions. 6.5 μ L of fractions 9, 12, 15, and 18 were analyzed by SDS-PAGE. B) 2.5 mg *E. coli* lysate was fractionated into 12 (pH 3-10) fractions. 6.5 μ L of fractions 3-10 were analyzed by SDS-PAGE.

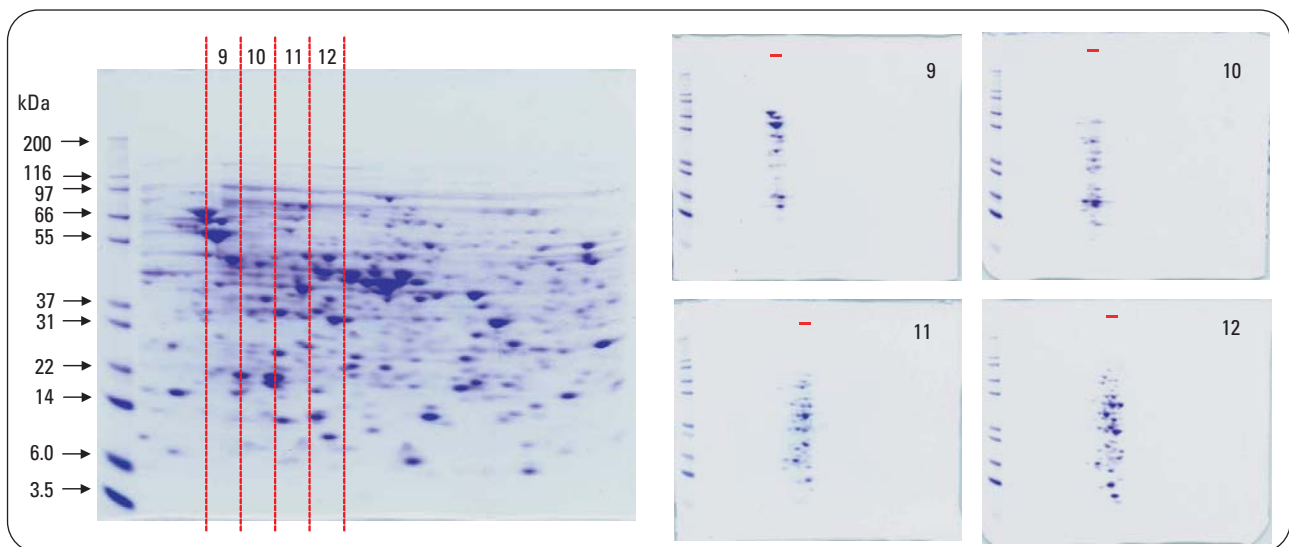


Figure 3
OFFGEL fraction analysis by 2DE. 1 mg *E. coli* lysate was fractionated into 24 (pH 4-7) fractions. 2D gels of fractions 9-12 (small gels, pH range 4-7) are shown. The target size of the OFFGEL fractions (0.11 pH/well) is indicated with a red bar. For comparison, a 2D gel of the unfractionated sample is shown as well (large gel, pH range 4.7-6.3). The approximate pH intervals corresponding to fractions 9-12 are indicated.

Results of the HPLC/Chip-MS analysis of a single OFFGEL run are shown in figure 4. The identified proteins show an even distribution across the fractions indicating an efficient reduction of sample complexity (figure 4a). One third of the proteins were found in only one or two OFFGEL fractions (figure 4b). The fact that proteins can spread across more than one fraction is not surprising since (i) proteins frequently have multiple charged isoforms due to variant post translational modifications, and (ii) identifications of processed or truncated protein variants that have often different pI values compared to the full length protein are merged into a single hit. As a comparison, after a fractionation of an *E. coli* peptide sample (tryptic digest of an *E. coli* lysate, pH range 3-10, 24 OFFGEL fractions) about 90 % of the peptides were identified in only one or two fractions^{2, 3}. After eliminating the redundancy between the OFFGEL fractions, i.e. counting proteins that are identified in more than one OFFGEL fraction only once, a total of 803 (5518) different proteins (peptides) were identified. This compares favorably to the results obtained with the unfractionated control sample that gave 225 (613) protein (peptide) identifications (figure 4c).

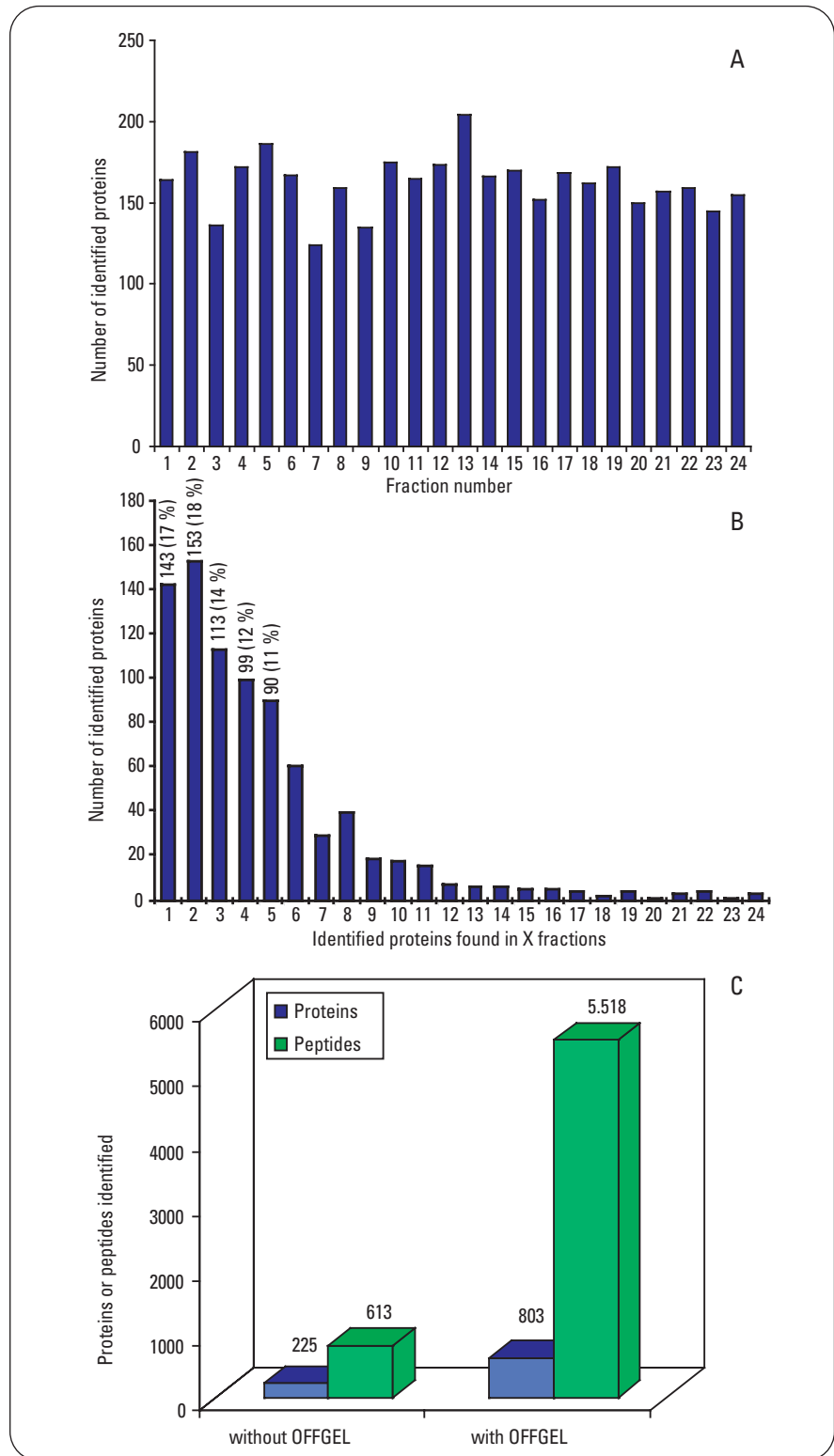


Figure 4
Analysis of *E. coli* proteins by OFFGEL electrophoresis and HPLC-Chip/MS. 1 mg *E. coli* lysate was fractionated into 24 (pH 4-7) fractions. A) Total number of proteins identified in each OFFGEL fraction, B) fraction-wise distribution of identified proteins, C) total number of non-redundant proteins and peptides respectively identified in OFFGEL fractions 1-24 and in a control without OFFGEL fractionation.

Conclusion

OFFGEL electrophoresis with the Agilent 3100 OFFGEL Fractionator offers easy recovery of the sample with excellent resolution and a good distribution of proteins across the fractions. The complexity is reduced in such a way that the mass spectrometer is used more efficiently enabling a deeper analysis of the proteomic sample. OFFGEL electrophoresis is a flexible technique for the pI based fractionation of proteins as well as peptides that can be used in various steps of multidimensional separation schemes.

References

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