Combining immuno-depletion, protein and peptide OFFGEL electrophoresis for the efficient fractionation of plasma prior to LC-MS analysis

Application

Patric Hörth
Christine A. Miller
Christian Wenz

Agilent Equipment

- 3100 OFFGEL Fractionator
- Multiple Affinity Removal System
- 1200 Series HPLC-Chip/MS System

Application Area

- Proteomics
- Protein Analysis

Abstract

The sample fractionation steps done prior to mass detection are of critical importance for the comprehensive analysis of complex protein mixtures. This Application Note illustrates the potential of combining protein depletion using the Agilent Multiple Affinity Removal System and protein or peptide fractionation using the Agilent 3100 OFFGEL Fractionator for the LC-MS based analysis of plasma proteins.

Agilent Technologies

Introduction

Comprehensive analysis of the human plasma proteome is challenging due to the complexity and wide range of protein concentrations. Efficient protein prefractionation is critical to detect low abundant plasma proteins. A well-established method to reduce sample complexity is the immuno-depletion of abundant proteins[1]. OFFGEL electrophoresis, on the other hand, fractionates proteins or peptides according to their isoelectric points[2]. Since the pI based fractionation of proteins and peptides, respectively, is orthogonal, both approaches can be combined in multidimensional separation schemes. With the Agilent 3100 OFFGEL Fractionator this can be done in parallel, as shown here for immunodepleted plasma. After depletion, human plasma proteins were either directly fractionated by protein OFFGEL electrophoresis, digested and analyzed by HPLC/Chip-MS. As an alternative, low abundant proteins were first digested, then fractionated by peptide OFFGEL electrophoresis and analyzed by HPLC/Chip-MS. Results from both experimental setups were complementary and allowed a more in-depth characterization of the sample.

Instrumentation

Immunodepletion

Lyophilized human plasma containing 3.8 % trisodium citrate as anticoagulant (Sigma, Taufkirchen, Germany) was reconstituted with water. For the depletion of the top six high abundant proteins the Multiple Affinity Removal system from Agilent Technologies was used as described elsewhere[3]. The fractions containing the low abundant proteins were pooled, concentrated and desalted by ultrafiltration using Vivaspin 4 concentrators (MWCO 5 kDa, Vivascience, Hannover, Germany). The sample was first concentrated to a volume of approximately 400 µl. Then, to desalt, 3.6 ml of 10 mM sodium phosphate buffer, pH 7.4 were added and the sample concentrated again. This step was repeated 3 times. Protein concentrations were determined with the Coomassie Plus Assay Reagent (Pierce, Rockford, IL, USA).

Protein OFFGEL electrophoresis

For pI based protein separation, the Agilent 3100 OFFGEL Fractionator with a 24-well setup (3100 OFFGEL High Resolution Kit, pH 4-7, Agilent part number 5188-6426) was used according to the protocol. One mg depleted human plasma was diluted with Protein OFFGEL Stock Solution, loaded and focused. For protein digestion with Trypsin, 5 µL 1 M ammonium bicarbonate and 10 µL 1 M iodoacetamide (freshly dissolved in 50 mM ammonium bicarbonate) were added to every fraction. After 30 minutes incubation in the dark, non-reacted iodoacetamide was quenched by adding 10 µL 1 M DTT. Fractions were desalted by gel filtration with 0.5 mL Zeba Desalt Spin Columns (Pierce, Rockford, IL, USA). As an alternative, the Agilent mRP-C18 High Recovery Protein Column can be used for rapid desalting and supplemental concentration of plasma proteins[4]. After adding 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.

Peptide OFFGEL electrophoresis

Depleted human plasma was reduced and denatured using 50 % 2,2,2-trifluoroethanol with 200 mM DTT at 95 °C for 20 minutes followed by alkylation with iodoacetamide at room temperature for one hour. The reduced and alkylated sample was diluted 1:10, Trypsin (Promega, Madison, WI, USA) was added at 1:20 enzyme:substrate and then incubated overnight at 37 °C. The digest was aliquoted, dried and stored frozen until use. For pI-based peptide separation, the Agilent 3100 OFFGEL Fractionator with a 24-well setup (3100 OFFGEL High Resolution Kit, pH 3-10, Agilent part number 5188-6424) was used according to the protocol. 200 µg sample digest were resuspended in Peptide OFFGEL Solution and loaded. After focusing, fractions were acidified with formic acid, analyzed directly, or stored frozen until use.

1- and 2-dimensional electrophoresis

1- and 2-dimensional electrophoresis were performed as described[5].

HPLC-Chip/MS analysis

For analysis by LC/MS, 10-20 % of each fraction was injected onto an LC/MS system consisting of an 1200 Series liquid chromatograph, an HPLC-Chip Cube MS interface, and an 6330 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 particels. Sample 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 µL/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 minute at a flow rate of 0.3 µL/min. The total run time of the gradient used, including column reconditioning was 65 min. The IPI Human database was searched with the Agilent Spectrum Mill Server software. An iterative searching strategy was employed for all searches. Autovalidation flagged the high confidence identifications so that no false positives are included.

Results and discussion

First, six high abundant proteins (albumin, IgG, IgA, haptoglobulin, transferrin and antitrypsin) were depleted from human plasma in a single chromatographic step. These proteins account for 80–90 % of the total protein content. The efficiency and specificity of the employed method has been shown elsewhere[1]. In the next steps, depleted human plasma was either directly fractionated by protein OFFGEL electrophoresis or first digested and then fractionated by peptide OFFGEL electrophoresis (figure 1). Protein and peptide OFFGEL electro-phoresis were performed in parallel by running the two sample sets on individual trays. As a control, an aliquot of depleted human plasma was digested and directly analyzed by HPLC-Chip/MS without prior OFFGEL fractionation (dotted line in figure 1). The quality of the protein OFFGEL fractionation was checked by one and two dimensional gel electrophoresis. The good reproducibility of the method was demonstrated by strikingly similar SDS-PAGE results of three independent OFFGEL runs (figure 2). Figure 3 shows the resolution of the method. Four neighbouring fractions of a single OFFGEL run were analyzed by 2DE. Proteins focused into sharp fractions with little overlap between neighbouring wells. HPLC/Chip-MS analysis results of the protein and peptide OFFGEL runs are shown in figure 4A. Compared to the control experiment without OFFGEL fractionation two- to threefold more proteins and three- to fourfold more peptides were identified in experiments including a protein OFFGEL or a peptide OFFGEL fractionation step. Three proteins with plasma concentrations between 10 and 100 ng/mL were identified in the peptide OFFGEL fractionation (table 1). This concentration range is typical for tissue leakage proteins[7].

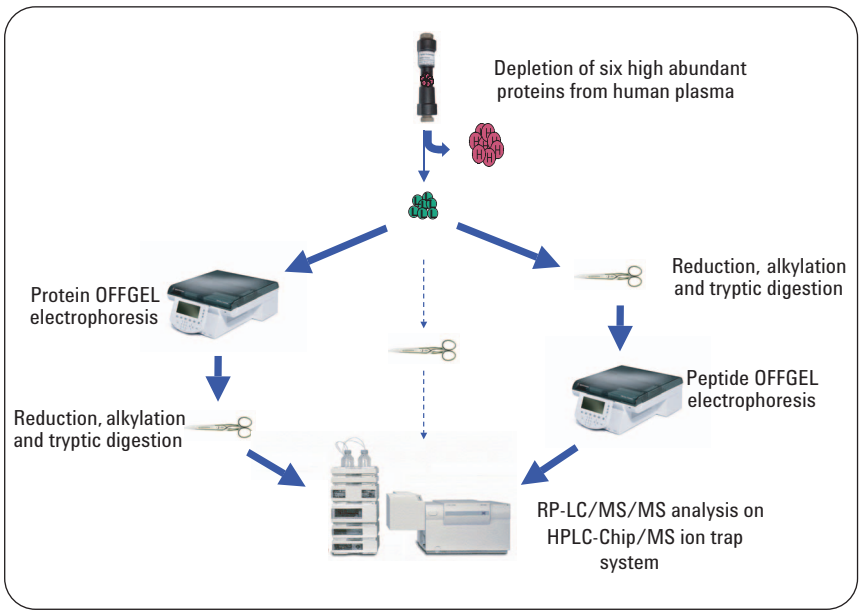

Figure 1
Experimental strategy.

Protein OFFGEL electrophoresis gave 16 % less protein identifications, but 24 % more peptide identifications compared to peptide OFFGEL electrophoresis. The lower number of protein hits could be due to the restriction of protein OFFGEL electrophoresis to the pH range 4-7, which excludes all proteins with pI values outside this pH range from the recovery and subsequent analysis. As an example, the low abundant insulin-like growth factor binding proteins 2 and 3, which both have pI values beyond 7, were not detected in the protein OFFGEL fractionation (table 1). In contrast to proteins that show a uniform pI distribution across the pH range 4-7 (data not shown

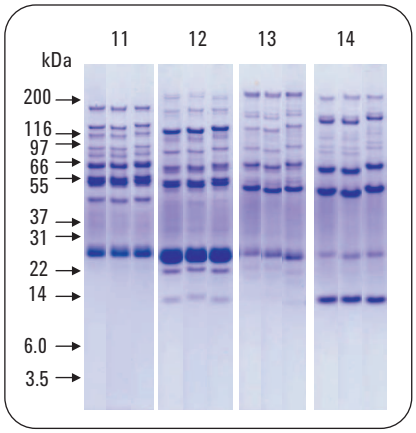

Figure 2
Reproducibility of protein OFFGEL electrophoresis. 6.5 µL of fractions 11-14 of three independent OFFGEL fractionations were analyzed by SDS-PAGE.

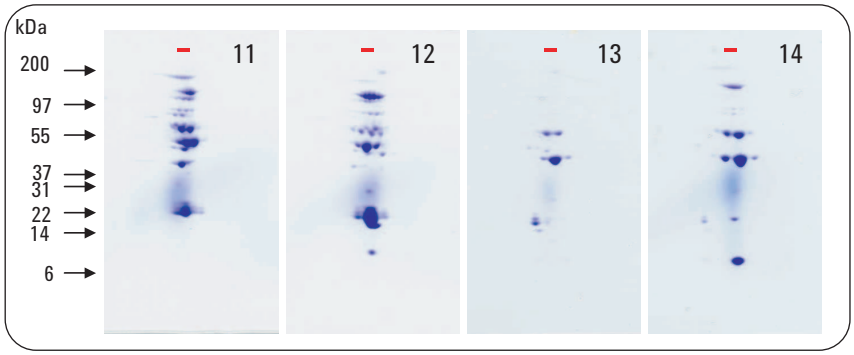

Figure 3
OFFGEL fraction analysis by 2DE (pH range 4-7). 2D gels of fractions 11-14 are shown. The target resolution of the OFFGEL fractions (0.11 pH/well) is indicated with a red bar.

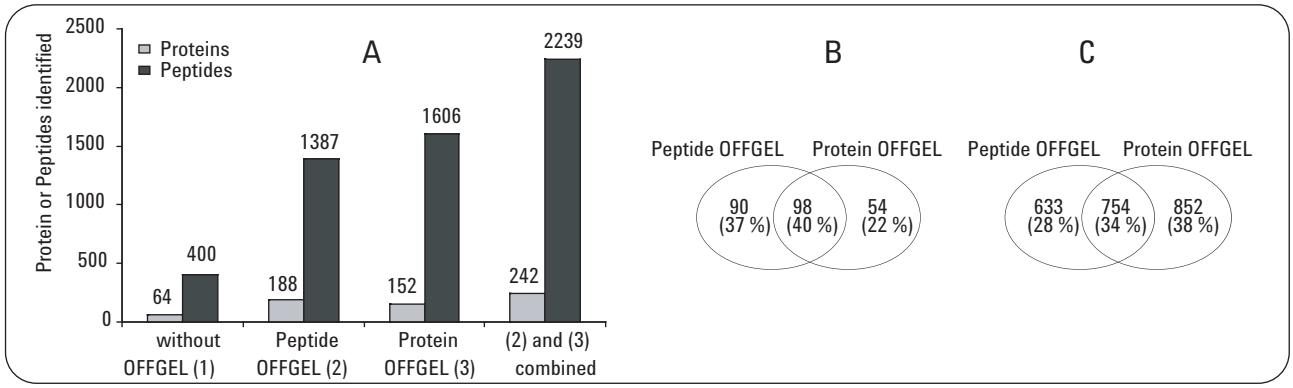

Figure 4
Analysis of human plasma proteins by immuno-depletion, protein and peptide OFFGEL electrophoresis and HPLC-Chip/MS. A) total number of non-redundant proteins and peptides respectively identified in a control experiment without OFFGEL fractionation (1), the experiment with peptide OFFGEL fractionation (2), the experiment with protein OFFGEL fractionation (3) and the combination of datasets (2) and (3). B) overlap of the protein identifications in datasets (2) and (3) and C) overlap of the peptide identifications in datasets (2) and (3).

and[5]), the isoelectric points of peptides cluster in specific pH ranges[2]. This indicates a more efficient reduction of sample complexity in the case of proteins and hence could explain the higher number of identified peptides in the protein OFFGEL compared to the peptide OFFGEL fractionation. The HPLC/Chip-MS results from protein and peptide OFFGEL electrophoresis respectively were complementary (figures 4B and 4C). Only 40 % (34 %) of the identified proteins (peptides) were present in both datasets. This overlap is much lower than the overlap of datasets produced by merely combining the MS results of two independent protein or peptide OFFGEL runs done under identical conditions (data not shown). By running two orthogonal separation techniques – protein and peptide OFFGEL electrophoresis – in parallel it was possible to substantially enhance the number of identified proteins and peptides (figure 4A).

Conc. ng/mL	accession_number	pI	entry_name
97	IPI00004373	5.39	Mannose-binding protein C
59	IPI00018305	9.03	Insulin-like growth factor binding protein 3
15	IPI00297284	7.48	Insulin-like growth factor binding protein 2

Table 1
Protein hits with plasma concentrations < 100 ng/mL. Concentration data were taken from Haab et al.[6].

Conclusion

The complexity of human plasma or serum can be efficiently reduced by a combination of immunodepletion and a pI-based fractionation on a protein or peptide level. OFFGEL electrophoresis is a flexible technique that can play a role in various steps of multidimensional separation schemes. Running protein and peptide OFFGEL electrophoresis in parallel can be a time and resource saving alternative to serial approaches.

References

1. Zhang K., Zolotarjova N., Nicol G., Martosella J., Yang L.-S., Szafranski C., Bailey J. and Boyes B., "Agilent Multiple Affinity Removal System for the depletion of high-abundant proteins from human serum – a new technology from Agilent." *Agilent Application Note 5988-9813EN,* **2003.**

2. Hoerth P., Miller C. A., Preckel T. and Wenz C., "Efficient fractionation and improved protein identification by peptide OFFGEL electrophoresis." *Mol. Cell. Proteomics 5, 1968-1974*, **2006.**

3. Vollmer M., Nägele E., Rüfer A. and Zhang K., "HPLC solution for the removal of high abundance proteins in human serum." *Agilent Application Note 5989-0263EN,* **2003.**

4. Barrett W., Martosella J., Boyes B., Szafranski C. and Nicol G., "A Rapid Method to Desalt and Concentrate Proteomic Samples Using the Agilent mRP-C18 Column." *Agilent Application Note 5989-2506EN*, **2005.**

5. Wenz C., Hoerth, P., "Efficient Protein Fractionation and Identification with the Agilent 3100 OFFGEL Fractionator" *Agilent Application Note 5989-5814EN*, **2006.**

6. Haab B. B., Geierstanger B. H., Michailidis G., Vitzthum F., Forrester S., Okon R., Saviranta P., Brinker A., Sorette M., Perlee L., Suresh S., Drwal G., Adkins J. N. and Omenn G. S., "Immunoassay and antibody microarray analysis of the HUPO Plasma Proteome Project reference specimens: systematic variation between sample types and calibration of mass spectrometry data." *Proteomics 5, 3278-91,* **2005.**

7. Anderson, N. L. and Anderson, N. G., "The human plasma proteome: history, character, and diagnostic prospects." *Mol Cell Proteomics 1, 845-67,* **2002.**

Patric Hörth and Christian Wenz are Research Scientists at Agilent Technologies Waldbronn, Germany. Christine A. Miller is Application Chemist at Agilent Technologies, Inc., Santa Clara, California, U.S.A.

www.agilent.com/chem/offgel

Published June 1, 2007,
Publication Number 5989-6419EN

Agilent Technologies