Multidimensional Intact Protein Separation and Comparative Visualization Strategy for Complex Analysis of Prostate Cancer Sera

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Introduction

The accurate fractionation, recovery, quantitation and characterization of individual proteins from complex proteomes are capabilities that are increasingly essential to the growth and success of biological research and education. The ability to characterize a complex protein sample by mass spectrometry depends on the power and sensitivity of the separation techniques employed prior to the MS analysis. The extreme complexity of such samples and the large dynamic range of protein concentrations demand a multi-dimensional separation strategy. Until recently, the analysis of whole proteomes had been heavily dependant on 2-Dimensional Gel Electrophoresis (2DGE) based approaches. However, this approach requires the laborious screening of hundreds to thousands of resolved "spots" on thin gels. The identification of even a small number of proteins of interest can require weeks to months to complete. More importantly, 2DGE provides poor resolution, irreproducibility and recovery of the intact proteins are limited.

In this study, we demonstrate a gel-free 2D separation strategy for comparative proteome analysis of prostate cancer and control patient sera. The methods involve affinity chromatography of the serum proteins, intact protein separation by use of Off-Gel™ isoelectric focusing followed by on-line reversed-phase chromatography fractionation and subsequent generation of comparative 2D visualization maps.

Methods

Sample Preparation
Sera of control and prostate cancer patient were obtained from Bioreclamation inc..

High Capacity Multiple Affinity Column
Agilent High Capacity Multiple Affinity Removal Column, 7.5mm i.d. x 100mm
120ml serum was diluted 4x with the loading buffer A for each depletion run. Sample was loaded onto the column and the flow-through collected, pooled, concentrated and diluted in Off-Gel Electrophoresis stock solution (thiourea, DTT and glycerol, pH 3-10 ampholyte).

ELISA
Standard sandwich enzyme-linked immunosorbent assays (ELISA) were used to determine the completeness of removal of targeted proteins from human serum.

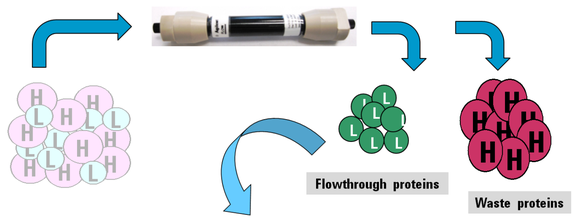
Isoelectric focusing (IEF)
IEF was performed by Off-Gel electrophoresis (Agilent 3100 OFFGEL fractionator) using 24 well (cm) strip with pH ranges from 3-10. All reagent and protein sample preparation was followed according to the OFFGEL Kit Guide. 1.5mg each depleted sera for control and patient were loaded onto the OGE instrument.

Reversed-phase Separation of IEF Proteins
24 IEF fractions from each sample type (control and prostate cancer patient) were RP separated by a high-recovery superficially macroporous reversed-phase column (mRP), 4.6 x 50 mm, (Agilent) under sample specific optimized high temperature (80° C) RP gradient elution conditions using a water (0.1% TFA)/acetonitrile (0.08% TFA) mobile phase. Proteins were monitored at 210 and 280 nm absorbance's. The IEF fractions were diluted 100% with water prior to LC injection. to decrease sample viscosity and no denaturant added (Fractions were denatured under IEF conditions). All RP work was performed on an Agilent 1100 HPLC equipped with automated fraction collector and thermosatted column oven.

Multidimensional Visualization Software
Prototype two-dimensional visualization software was developed by Agilent (MDVis, version 0.3.1.1). MDVis provides a visual analysis environment for multidimensional separations and comparative proteome analysis.

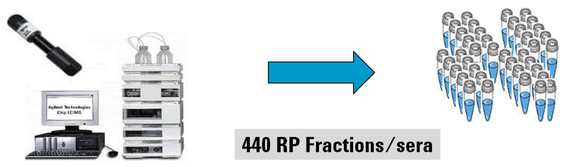
Experimental Workflow

Immunodepletion Chromatography – 14 High Abundant Proteins removed

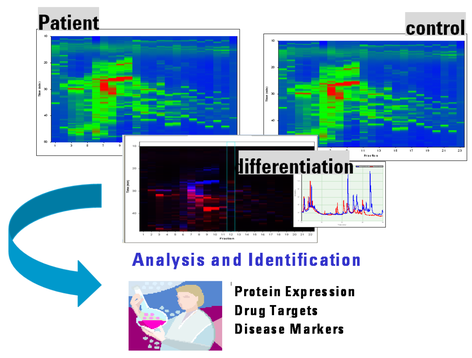


1st Dimension Separation – Off-Gel Electrophoresis

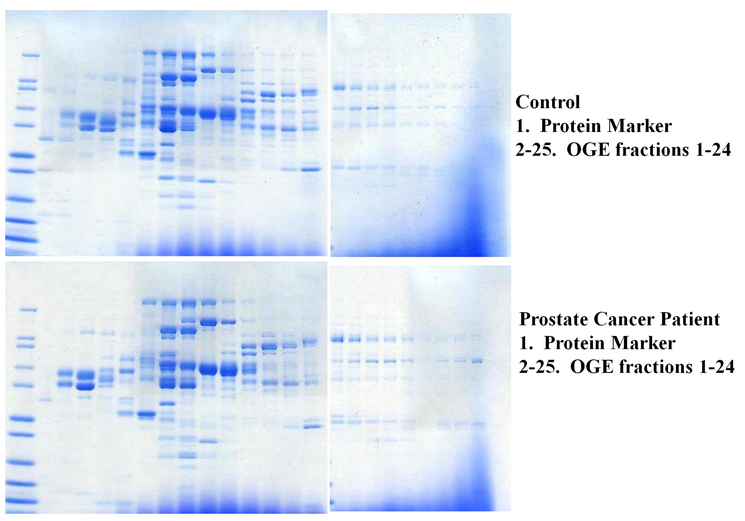
2nd Dimension Separation – Hi Temp RP Separation



Multidimensional Visualization & Differential Proteome Identification



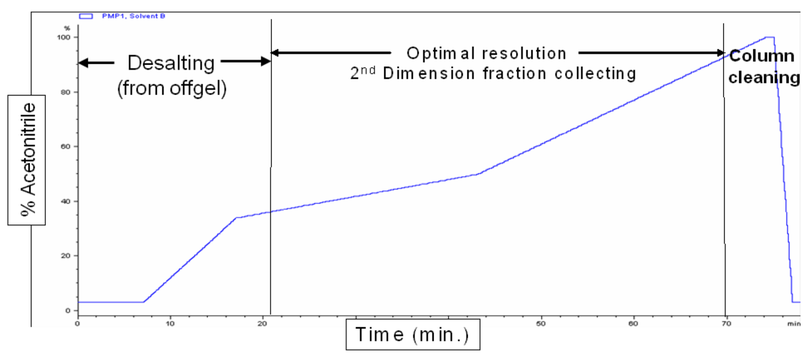
IEF SEPARATION



SDS-PAGE 4-20% gel image comparison of IEF separation between control and prostate cancer sample type. 5ul sample from each of 24 OGE fractions were mixed with 5 ul sample buffer and loaded onto a 15 well SDS-PAGE 4-20% gel.

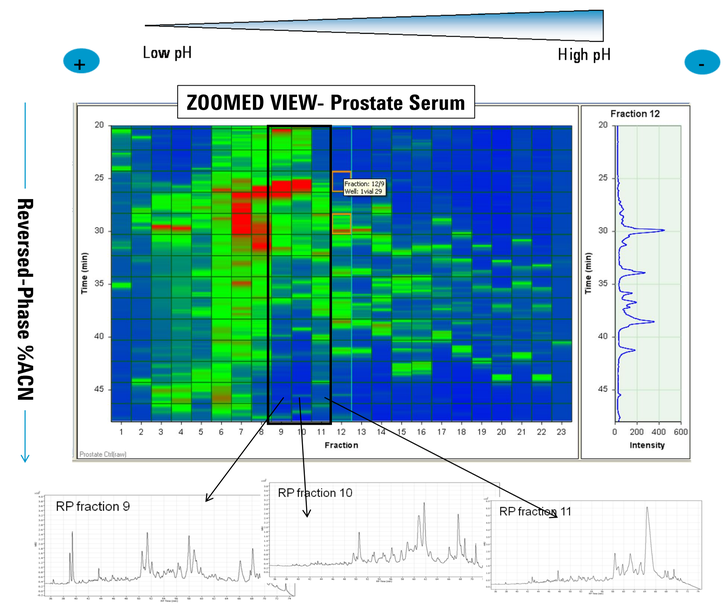
Reversed-Phase Separation

Optimized RP Gradient Conditions



Sample: Prostate cancer immunodepleted serum proteins (high salt /5.0% AcOH) after IEF separation
Mobile Phase & Conditions: A-0.1% TFA/water, B-0.08% TFA/ACN, multi-segmented gradient, flow 0.75ml/min (salt elution) to 5.0 min, 0.25mL/min to 60min,.75 ml/min 60-70min.,Temp. 80 C, DAD 210 & 280nm.

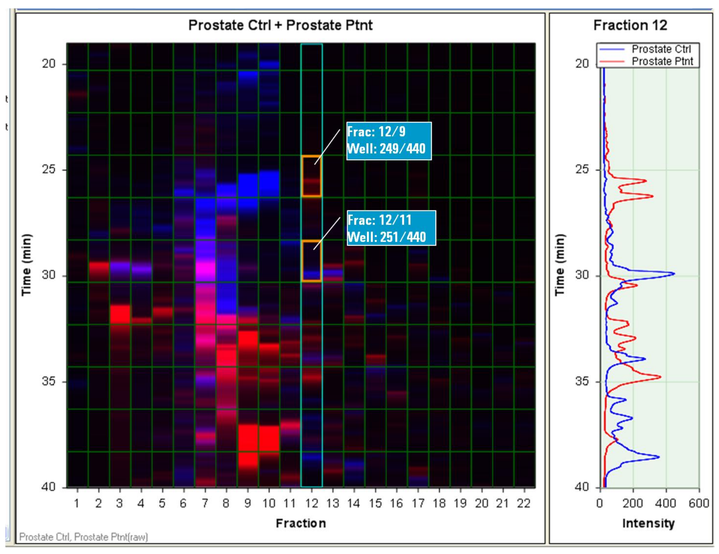
IEF x RP 2D MAP



Zoomed View 2-D map of IEF x RP separation of Prostate Cancer Serum. 20 RP fractions/24 RP runs were collected at 2-minute time slices from 8-48 min. A total of 480 fractions were collected per sample set (control and diseased sera) for selective HPLC-chip/MS analysis (data not shown-phase 2 project).

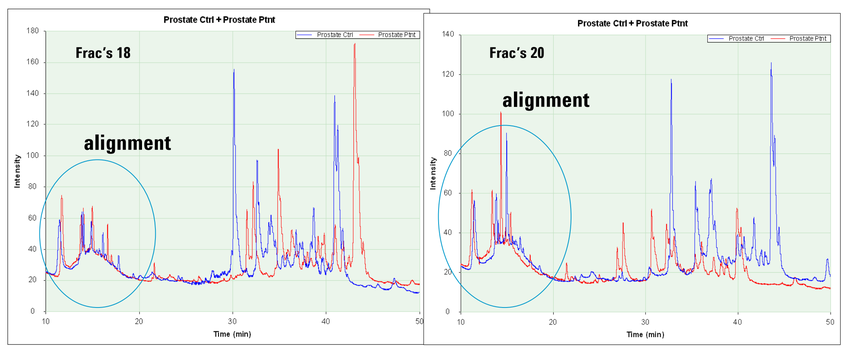
Comparative Proteome Analysis

Prostate Cancer Serum/Healthy Serum 2D Differential Analysis



A differential overlay view of prostate cancer and healthy (control) patient serum has been created after superimposing both sample sets. The first data set is displayed in blue and the second data set in red, using the same colors as the side chromatogram. Colors are blended so if a feature is unique to a single data set it will show as pure red or pure blue. When peaks overlap they are displayed as purple (some overlap) or black (identical peaks).

Algorithmic Alignment Between Sample Sets for Accurate Fraction Selection



Alignment of the RP separations in each set, and between two sample sets , are critical for selecting and analyzing fractions that indicate potential changes among the proteomes. Correct alignment ensures a higher degree of confidence for choosing fractions which display absorbance variations.

Conclusions

The materials, methodology and comparative 2-D maps demonstrate utility to rapidly identify samples for comparative protein identity by LC/MS analysis. We believe this strategy shows promise as a proteomic platform for rapid comparative studies of human disease states and additionally has the potential to indicate protein modifications and /or protein isoforms that can elute at an altered position in the 2-D plot. It is the authors intentions to complete HPLC-chip/MS analysis among the 960 fractions collected that indicate an expression change among the diseased and healthy proteomes (phase 2).