Biomarker Proteins in Diabetic and Normal Serum Samples Using Both Chromatographic and Preparative Electrophoretic Fractionation With 2-D Gels

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Introduction

Biomarker discovery and validation is an important part of most proteomics programs in academia and the biotech industry. The goal is to identify and verify up- or downregulated proteins that are part of a disease-specific pathway and thus allow early diagnosis. Typically, biological material of a disease sample and a control sample are subjected to fractionation, separation, and quantitative analysis to determine possible markers. In clinical and pharmaceutical studies, serum and plasma are the preferred samples since they can be obtained in a noninvasive and routine way, stored for weeks or months, and contain a plethora of biologically relevant information on the whole organism, mostly in the form of proteins and protein degradation products.

Currently three major approaches to separate and identify protein biomarkers are pursued: 1) 2-D gel separation followed by image analysis, spot cutting, digestion, and mass spectrometric (MS) identification; 2) bottom-up proteomics, where a more or less complex protein mixture is first digested into an even more complex tryptic peptide mix before being separated with 2-D high-performance liquid chromatography (HPLC), and online MS identification; 3) top-down proteomics, where the intact protein mixture is separated via chromatographic or electrophoretic methods prior to high-resolution MS identification with Fourier transform ion cyclotron MS (FT-ICR-MS). In this poster, we concentrate on the first methodology. See Figure 1 for an overview of the experimental design.

Blood, serum, and plasma have been research subjects since the beginning of modern medicine. Therefore, a lot is already known about these samples. However, the 12 most abundant proteins found in a serum sample make up 99.4% of all proteins in the sample. These well-known proteins are neither over- nor underexpressed in a disease state, and therefore will not be biomarkers. The challenge in using serum as a sample in a biomarker study is to sufficiently fractionate out the most abundant proteins to enable the discovery of a lower-abundance differentially expressed protein. Here we propose a strategy using chromatographic and electrophoretic methods to fractionate diabetic serum and a control serum prior to 2-D gel electrophoresis, tryptic digestion, and protein identification with an ion-trap MS-MS approach.

Results and Discussion

The workflow to separate and detect low-abundance proteins in serum begins with the chromatographic removal of the most abundant proteins, albumin and IgGs, followed by a Rotofor® cell fractionation and a 2-D gel separation with micro-range ReadyStrip™ IPG strips (Figure 1). An automated preparative two-column setup with a 450 ml Affi-Gel® Blue column and a 10 ml protein A column in series was used to separate crude serum into three fractions: general serum proteins, an IgG fraction, and an albumin fraction (Figure 2). We injected and fractionated 10 ml each of a pooled diabetic sample and a pooled normal control into 30 ml collection vials. The chromatographic trace for both samples was identical with respect to the non-IgG and non-albumin proteins collected (Figure 2, tubes 3 and 4). Those fractions were combined, and 18 ml for each of the diabetic and normal samples was subjected to a mini Rotofor cell run (Figure 3), which resulted in 20 fractions of 700 µl each. Figure 4 indicates the reproducibility of the preparative isoelectric focusing. Especially in the acidic range, the pH can shift as much as 1 pH unit for a given fraction. We therefore also measured the protein content and aligned the fractions according to their pH (Figure 5); this results in similar patterns for the diabetic and normal serum samples. The pH values of the Rotofor fractions were used to determine which were pooled for the first-dimension separation on micro-range ReadyStrip IPG strips.

Three 2-D gel separations are shown for each sample, covering the pH ranges of 3.9–5.1, 4.7–5.9, and 5.5–6.7 (Figure 6). These images were used for PDQuest™ software analysis (Figure 7). Compared to a 2-D gel covering a pH range of 3–10, after the chromatographic depletion, the number of spots in the 2-D gel increased 4-fold (Table 1). More importantly, the number of differentially expressed proteins, prime candidates in the search for biomarkers, increased by an order of magnitude from 1 to 20.

A number of proteins, both differentially expressed and with equal quantity, were subjected to digestion with trypsin and MS analysis using a nanoflow HPLC-MS approach. Table 2 shows the results of a selected group of IDs from both normal and diabetic sample gels. This work in progress does not yet show any surprising candidates for a diabetic biomarker, but the identified glycosylated proteins are upregulated in the diabetic sample. An effort to identify all 58 candidates found with this approach is underway.

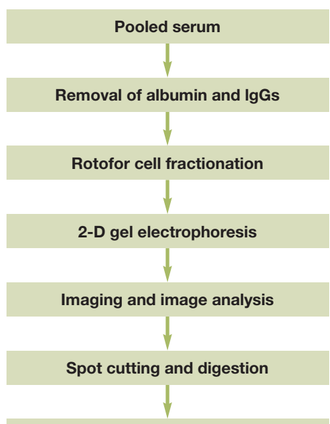

Fig. 1. Experimental design.

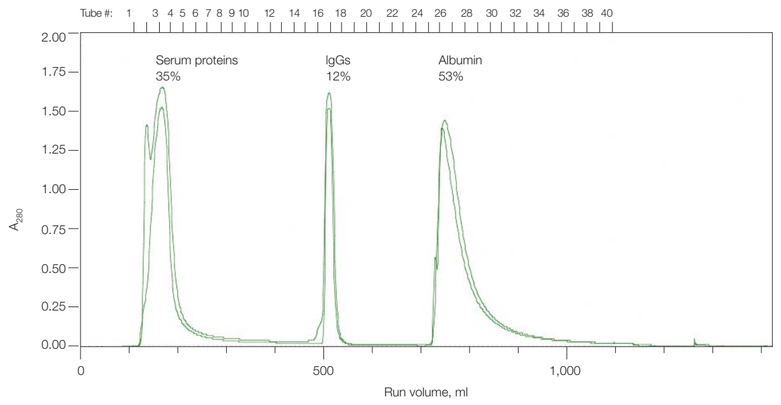

Fig. 2. Preparative chromatography. Automated two-column operation with Affi-Gel Blue and protein A columns in series to remove albumin and IgGs.

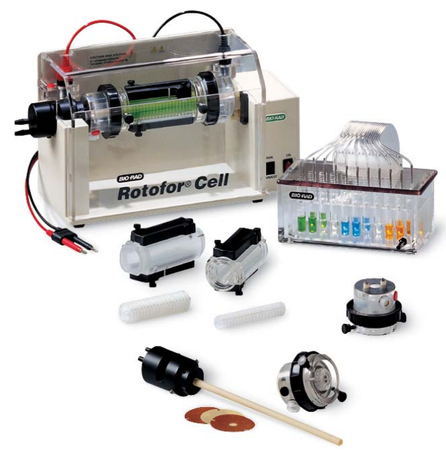

Fig. 3. The Rotofor cell used to fractionate serum.

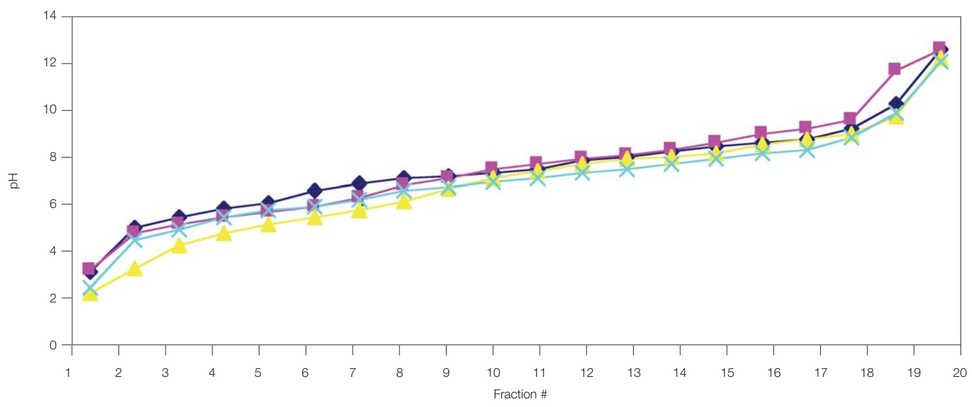

Fig. 4. Reproducibility of preparative isoelectric focusing with the Rotofor cell.

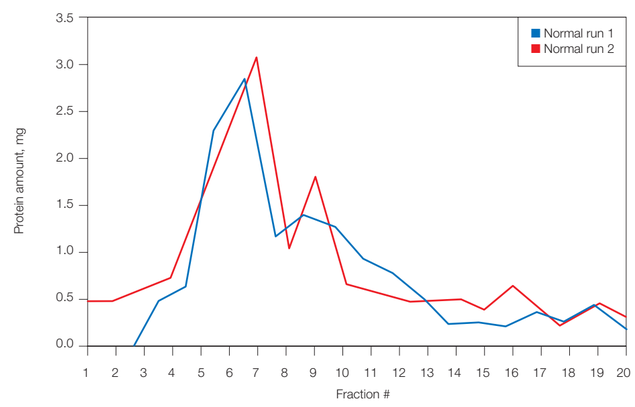

Fig. 5. Protein recovery.

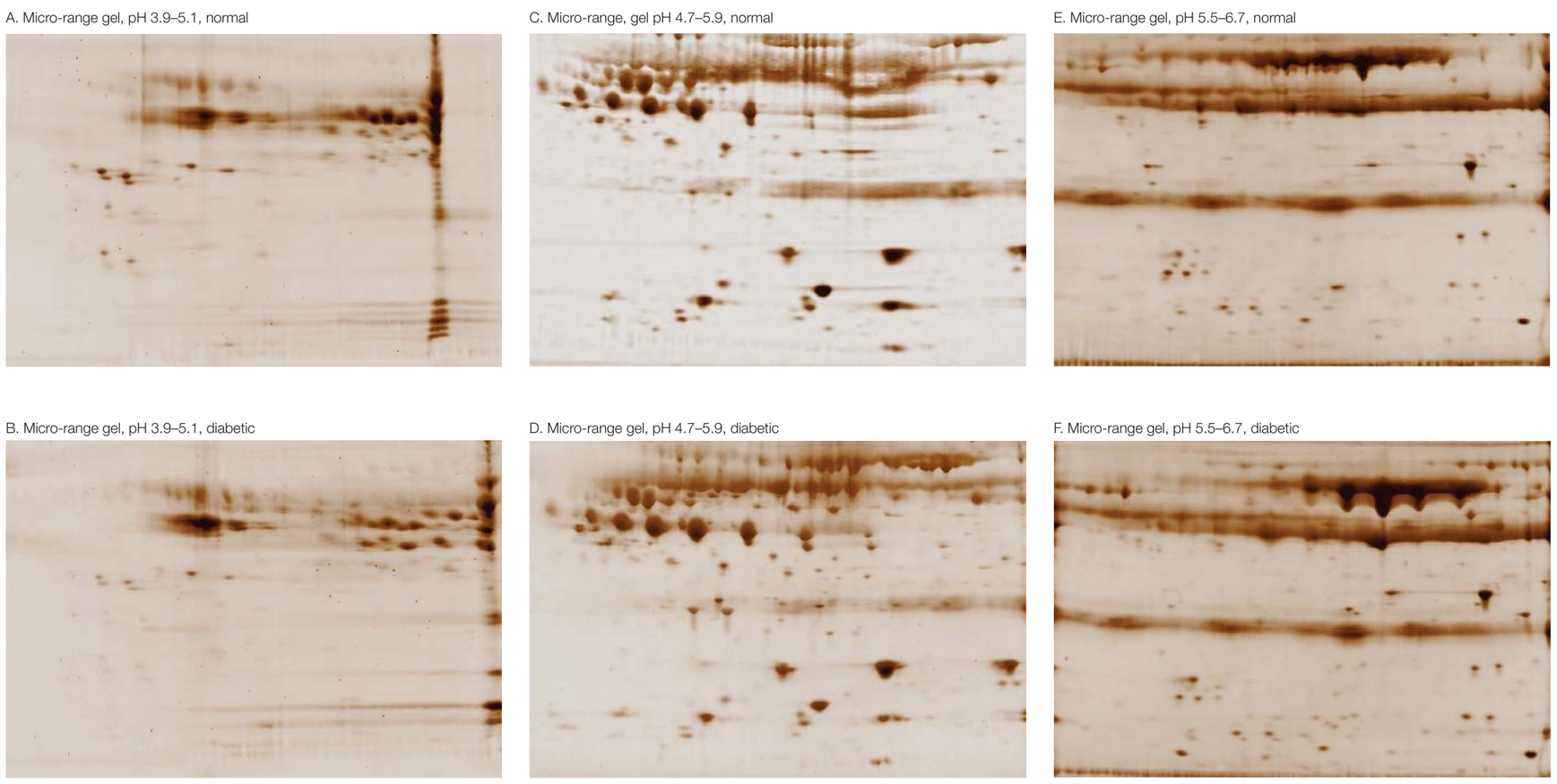

Fig. 6. 2-D gel images of micro-range separations.

Table 1. PDQuest analysis: diabetic and normal serum 2-D gel spot counts with and without fractionation enrichment.

2-D Gel Type	Diabetic Serum	Normal Serum	Unique Spots	≥ 2-Fold Change in Expression
pH 3–10 strips	146	163	1	4
Rotofor cell fractionation + pH 3.9–6.7 strips	593	612	20	38

Table 2. MS ID results.

Spot	Source	P	XC	% Coverage	Mass	Database pI	Database Mass	Gel pI	Gel Identity
A3	Diabetic	2.8×10^{-6}	60.2	26.87	23	4.25	75	5.1–5.2	Apolipoprotein chain A
A5	Diabetic	1.3×10^{-6}	78.2	13.33	54	6.0	27	5.2	α1B-glycoprotein
A6	Diabetic	2.5×10^{-7}	46.2	7.07	54	6.0	50	5.3	α1B-glycoprotein
A7	Diabetic	3.4×10^{-10}	176.2	69.15	23	4.3	80	5.5	Apolipoprotein chain A
B4	Normal	1.4×10^{-6}	40.1	23.36	23	4.3	80	5.5	Apolipoprotein chain A
B5	Normal	6.7×10^{-15}	10.3	35.4	7	6.0	20	4.8–4.9	Hypothetical protein
B6	Normal	1.4×10^{-6}	48.2	9.7	54	6.0	20	5.0–5.1	α1B-glycoprotein

Conclusions

- The workflow for a biomarker discovery program using blood serum or plasma and based on chromatography, isoelectric focusing, and 2-D gel electrophoresis has been validated
- Preparative 2-column chromatography removed about 65% of protein mass with IgGs and albumins
- Chromatography was highly reproducible for 10 ml serum injections of both diabetic and normal control sample
- Rotofor cell and subsequent pooling based on fraction pH and pH range of ReadyStrip IPG strips is an efficient tool for further sample fractionation
- A 4-D approach resulted in a larger number of differentially expressed proteins, increasing the odds of finding a valid biomarker
- Initial identification showed glycosylated proteins upregulated in the diabetic sample
- The MS ID program as well as validation of identified proteins with respect to diabetes are ongoing