

Glycoproteome Changes in Breast Cancer: Identification by Multi-lectin Affinity Chromatography (M-LAC) combined with *digital* ProteomeChip™ (dPC™) and Mass Spectrometry

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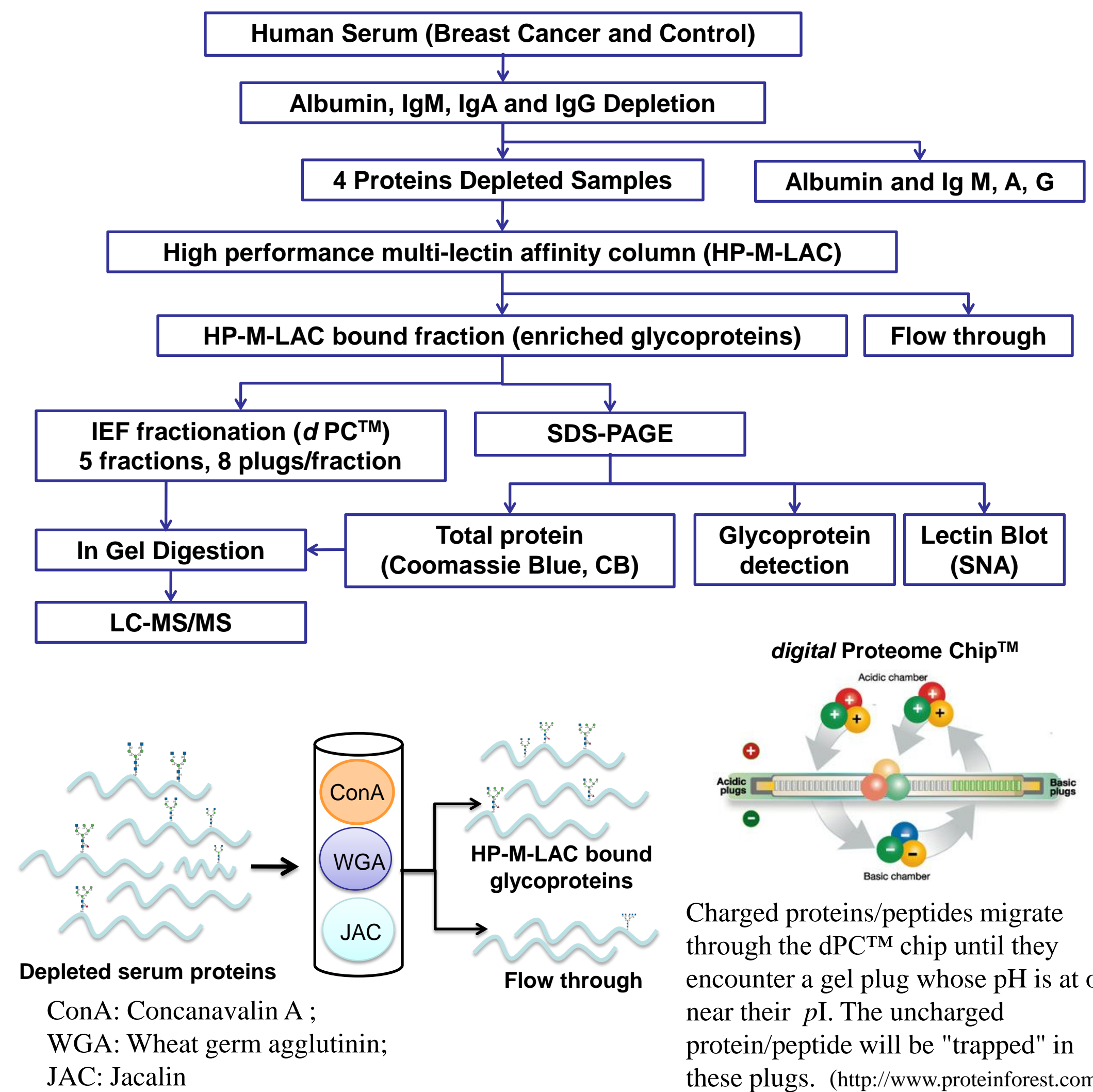
Abstract

Protein glycosylation represents one of the major post-translational modifications and can have significant effects on protein function. Moreover, change in the carbohydrate structure is increasingly being recognized as an important modification associated with cancer. In this presentation we describe the development and application of a proteomics platform to measure changes in the serum glycoproteome of females with breast cancer. Diseased and healthy serum samples were processed by an optimized sample preparation protocol that partitions serum proteins based on glycan characteristics with the goal of identifying changes in either the concentration level and/or the carbohydrate structure of the glycoprotein(s). The method involves immunodepletion of abundant proteins, selective glycoprotein enrichment using M-LAC and fractionation by isoelectric focusing (IEF) using the *digital* ProteomeChip™ (dPC™). Peptide sequencing and protein identification were accomplished by LC-MS/MS and data was analyzed using the Mass Spec Results Analysis Tool™ (MSRAT™) bioinformatics software. A comparison of cancer and control samples revealed that this approach increased the information content of the analysis; both in terms of identifying changes in protein concentration of circulating glycoproteins, and detection of differences in glycoform profiles. We will also present data that shows the utility of this proteomic platform for deeper mining of the serum glycoproteome and for the simultaneous profiling of glycosylation and glycoprotein abundance. In addition, we will discuss the identification of several potential glycoproteins that distinguish cancer from control samples and are candidates for future biomarker validation studies.

Methods

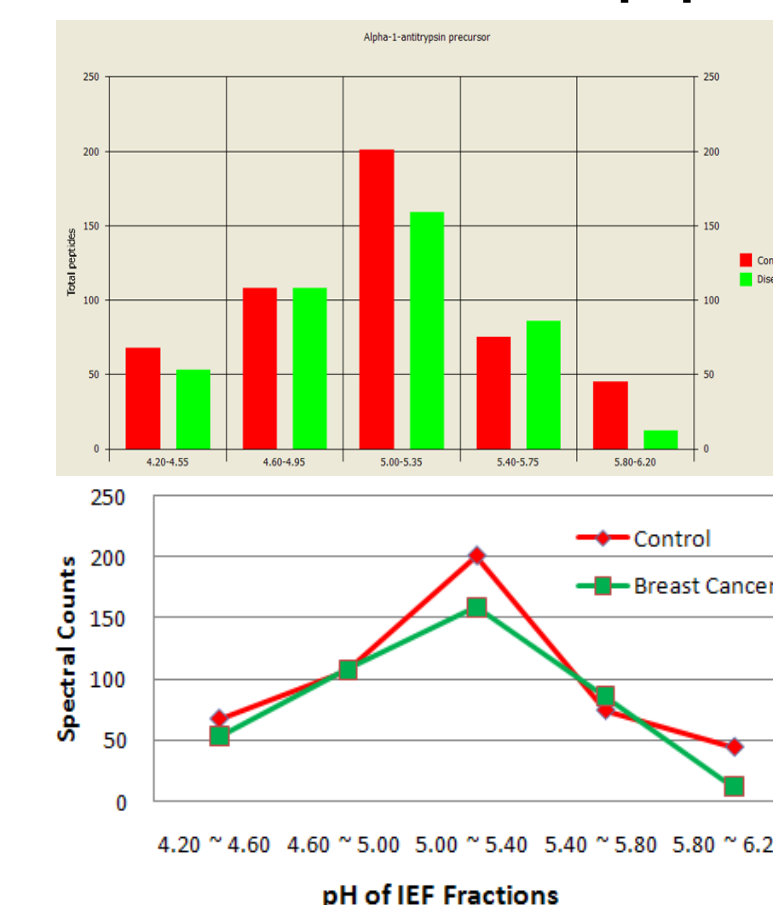
- We used human serum from 5 different anonymous control (matched by gender, race and age) and 5 breast cancer (stage 2) donors. For these studies, equal amounts of each serum sample were combined to make one control and one disease.
- High abundant proteins (albumin, IgG, IgM and IgA) were immunodepleted
- Glycoproteins in the depleted serum were enriched using high performance multi-lectin affinity column (HP-M-LAC), containing a mixture of 3 lectins. The bound fraction was analyzed by SDS-PAGE; total proteins were visualized by Coomassie blue, and glycoproteins were detected on gel by fluorescence (periodic acid-Schiff reaction), or in blots by probing with biotinylated *sambucus nigra lectin* (SNA). SNA binds preferentially to sialic acid attached to terminal galactose in (α -2,6).
- HP-M-LAC enriched glycoproteins, were further fractionated based on charge using the *digital* Proteome Chip™ (dPC™).
- Proteins were digested with trypsin and the resulting peptides were separated using C18 reversed phase chromatography. The LC-MS/MS experiments were performed on a nano-HPLC system (Eksigent) coupled to a LTQ ion trap. MS/MS spectra were processed using the Sequest search engine. Data analysis was performed using Mass Spec Results Analysis Tool™ (MSRAT™).

Workflow



Results

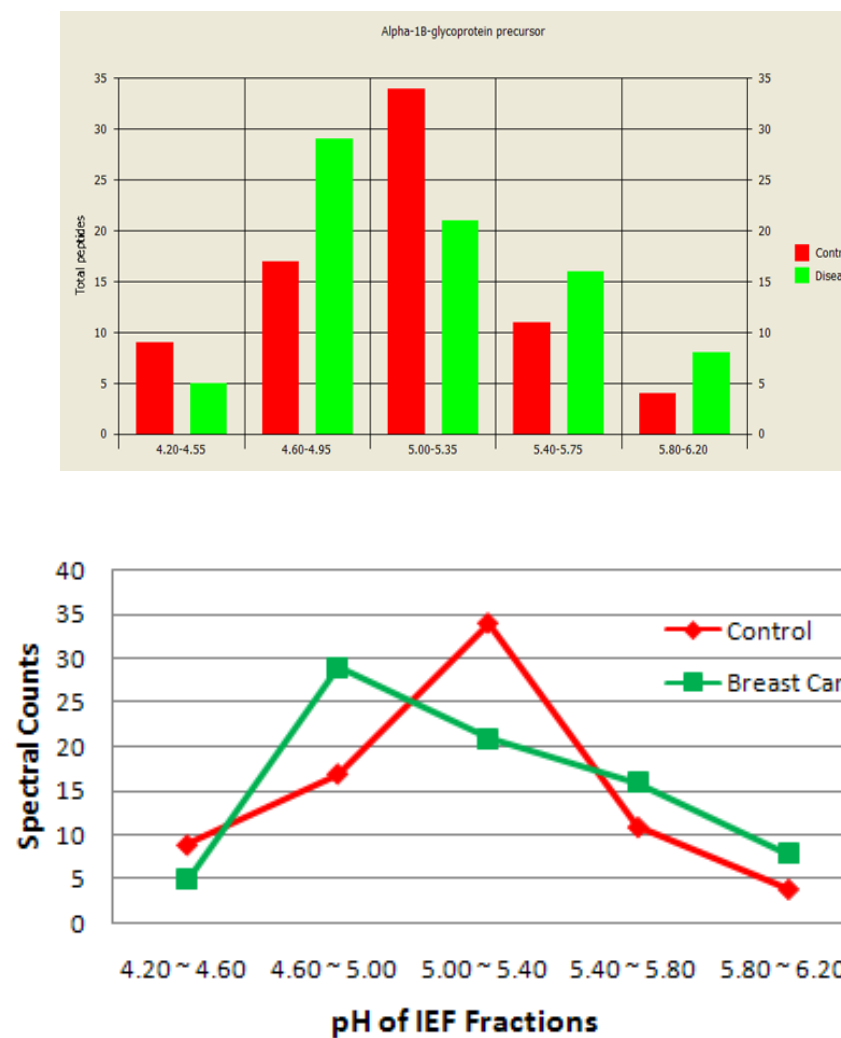
- The levels of alpha 1-antitrypsin (band 17 on SDS-PAGE) was found to decrease in breast cancer compared to control. This was suggested by the number of total peptide hits and all the gel staining methods.



dPC Fractions	Control	Breast Cancer
4.20 ~ 4.60	9	5
4.60 ~ 5.00	17	29
5.00 ~ 5.40	34	21
5.40 ~ 5.80	11	16
5.80 ~ 6.20	4	8
Total	75	97

Based on spectral counts (total peptide hits), the abundance of alpha-1-antitrypsin is decreased by about 20%. Data was analyzed by MSRAT.

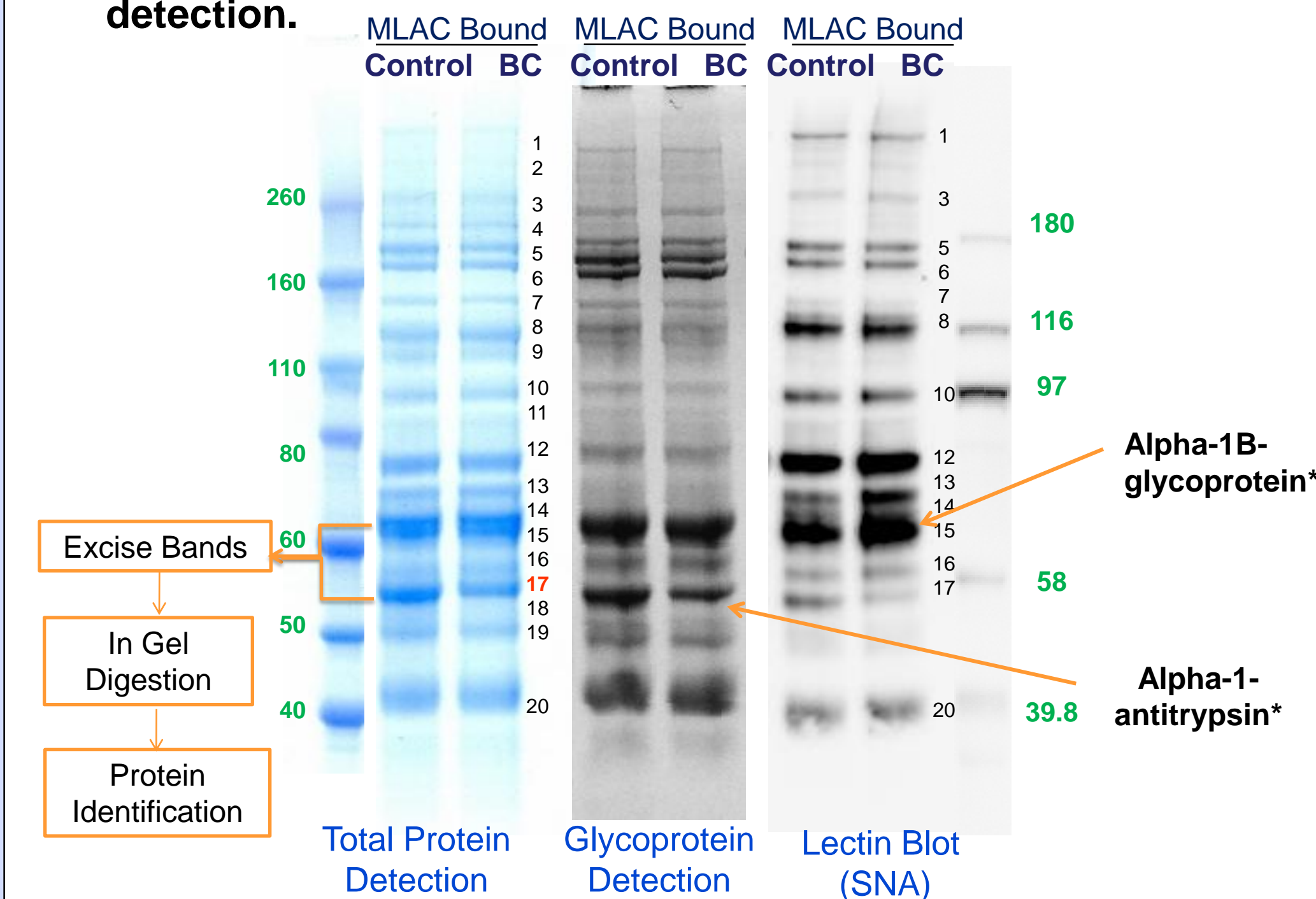
- Band 15 isolated from SDS-PAGE was identified as alpha-1B-glycoprotein. The lectin blot and dPC™ results suggests potential changes in glycosylation.



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5.80 ~ 6.20	4	8
Total	75	97

Spectral counts of alpha-1B-glycoprotein identified in each IEF fractions. A shift towards the acidic region is observed for breast cancer. This is consistent with the increased binding observed with the SNA lectin, suggesting that the amount of sialic acid changes in breast cancer.

- Selection of proteins for proteomic analysis based on differences observed from glyco-staining and lectin blot detection.



* Proteins with the highest peptide hits in that band.

Glycoproteins enriched by HP-M-LAC were analyzed by SDS-PAGE followed by different detection methods. Band 15 and 17 were excised and analyzed by LC-MS/MS. The top ranked protein having a molecular weight in the expected range (correlated to SDS-PAGE) was taken as the most likely candidate. Validation of protein identity by immunoprecipitation and Western blotting is underway.

- LC-MS/MS Proteomic Analysis: Partial list of identified proteins before (-) and after (+) fractionation by dPC™

Protein	+ IEF (Pep Hits)		- IEF (Pep Hits)	
	Control	Disease	Control	Disease
Cadherin-5	2	3	0	0
Transforming growth factor-beta-induced protein ig-h3	5	3	0	0
Pigment epithelium-derived factor	7	8	6	4
Serum paraoxonase/arylesterase 1	46	45	1	1
Thrombospondin-1	36	30	0	0
Insulin-like growth factor-binding protein complex acid labile	64	52	2	1
Apolipoprotein D	17	26	1	4
Apolipoprotein M	3	1	0	0
Kallistain	39	48	1	3
Apolipoprotein-L1	3	1	1	0
Monocyte differentiation antigen CD14	7	5	1	0
Tetranectin	2	2	1	1
Corticosteroid-binding globulin	7	4	0	0

Proteins (medium level) identified by HP-M-LAC enrichment, and with (+) and without (-) dPC™ fractionation. We observed a "deeper mining" of the serum glycoproteome with the downstream addition of dPC™ to this workflow.

Conclusions

- Established a robust workflow to characterize the serum glycoproteome and identify breast cancer-induced glycosylation changes.
- The lectin blotting assay has been used as a tool to detect proteins with potential glycosylation changes between breast cancer and control. Protein bands which show differences in the staining intensity are excised from the gel for further characterization by LC-MS/MS analysis. The sialic acid (SNA) lectin was used as an example in this study; a panel of other lectins are currently being evaluated.
- The dPC™ charge-based fractionation technology was easily coupled with abundant protein depletion and glycoprotein enrichment. Our analysis showed an improvement in the dynamic range of protein identifications.
- The use of the MSRAT bioinformatic tools enhanced our abilities to rapidly compare case and control for changes in protein abundance and glycoform differences. A list of potential candidates has been compiled for further evaluation.