Automated Profiling and Identification of Endogenous Peptidomic Markers in Human Plasma

T. Richmond[1], M. Askenazi[1], J. Sutton[1], L. Bonilla[1],

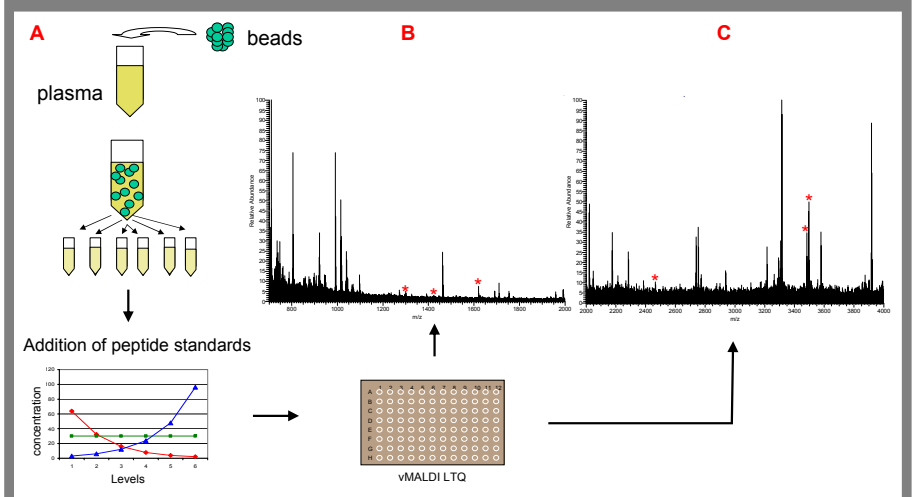
[1]Thermo Electron BRIMS Center, Cambridge, MA, USA

Purpose: Develop an automated, vMALDI-based method and software algorithm for differential profiling of endogenous peptides in human plasma.

Methods: A robotic system was employed for transferring and step-wise manipulation of hydrophobic, surface-activated magnetic beads to selectively separate and enrich endogenous peptides found in human plasma. A new differential expression software (BioSieve™) was developed to automatically identify statistically significant changes in relative signal intensity in vMALDI MS spectra. The overall precision of the method to bind and detect changes in peptide levels was characterized using a mix of six synthetic peptide standards spiked into human plasma.

Results: Using this platform we were able to perform trend analysis to track changes in the levels of individual peptides within a complex mixture. The automated fractionation process combined with vMALDI and HT data processing reproducibly enriched spiked and endogenous peptides preserving the relative abundance information and allowing high-throughput differential analysis at low fmol levels.

FIGURE 1. (A) Work flow, (B) normal range (700-2000 *m/z*), and (C) high-mass range (2000-4000 *m/z*) vMALDI MS spectra of human plasma enriched using hydrophobically-coated magnetic beads. Asterisks denote spiked standard peptides.

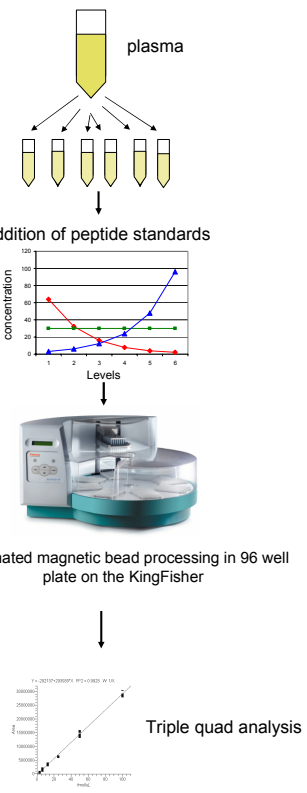


Introduction

Interest in biomarkers has experienced an explosion in recent years. A lot of attention is being paid to the potential clinical application of using well characterized changes in MS patterns of the peptidome as indicators (predictors) of various disease states. The peptidome of human plasma has been estimated to contain ~5000[1] unique peptides. Development of robust methods that include the accurate identification of the relevant components of the peptidome is critical not only to our understanding of the biology of disease states, but also to our ability to discover markers for these states.

Methods and Results

Automated Sample Preparation – To simulate a "time-course" experiment in human plasma, Angiotensin I, fibronectin fragment (1377-1388), bombesin, ACTH fragment (18-39) glucagon and oxidized insulin B chain (Sigma, St. Louis, MO) were spiked together into thawed human plasma at varying concentrations as follows: two peptides decreasing two-fold per step, [angiotensin I (640-20fmol/μL), glucagon (64 - .2pmol/μL)], two peptides increasing two-fold per step [fibronectin (30-960fmol/μL), ACTH (80-2560fmol/μL)] and two peptides held constant [bombesin (300fmol/μL) and Insulin B chain oxidized (1600fmol/μL)] yielding a total of six plasma samples as shown at right. Spiked plasma was kept on ice until fractionation. Each of the six different levels were added to a row on a 96-well plate (level 1 into row A, level 2 into row B etc.) at 25μL per well. A row of unspiked plasma was added as a negative control. The 96-well plate was processed on the KingFisher 96™ (Thermo Electron, Vantaa, Finland) to capture and enrich endogenous and spiked peptides. The KingFisher uses magnetized rods to collect and move the beads from well to well.



50mL of beads were used per well with a contact time of 2mins followed by 3 washes of 30 seconds each during which time the beads were released into wells containing 0.1% formic acid in water and agitated at a slow speed. The beads were then collected and moved to wells containing fresh washing solution. To elute the peptides, the beads were moved to wells containing 25μL of 50% acetonitrile with 0.1% formic acid. The beads were collected by the KingFisher and removed from the eluant. One half microliter was spotted on a MALDI plate sandwiched between 0.5mL of CHCA (Sigma, St. Louis, MO). Data was collected on a Finnigan™ LTQ™ linear ion trap fitted with a vMALDI™ source (Thermo Electron, San Jose, CA). For each sample, full scan spectra were collected in centroid mode over two separate *m/z* ranges (700-2000 and 2000-4000). Peptide recovery was monitored using a fast LC-SRM assay on a Finnigan TSQ Quantum ™ HR triple quadrupole MS (Thermo Electron, San Jose, CA).

Software Development – Following magnetic bead fractionation, the bound fraction of human plasma was spiked with increasing concentrations of ACTH (8-256fmol/μL), decreasing concentrations of glucagon (640-20fmol/μL) and a constant level of oxidized insulin B chain (160fmol/μL) to produce a total of six different spike levels (Figure 1). Each of the six levels was spotted 11 times resulting in a total of sixty-six MS spectra. vMALDI MS data was collected as described above to test BioSieve, developed in part, to automatically profile relative changes in the MS patterns among multiple LC/MS sample sets (Figure 3).

FIGURE 2. (A) Calibration curve used for the LC-SRM quantitation of fibronectin on the Finnigan TSQ Quantum (679.15 *m/z*, 2+ → 619.41 *m/z*, -21 V, 1.5 mTorr). (B) Increasing recovery of fibronectin peptide from plasma with increasing concentration of spike.

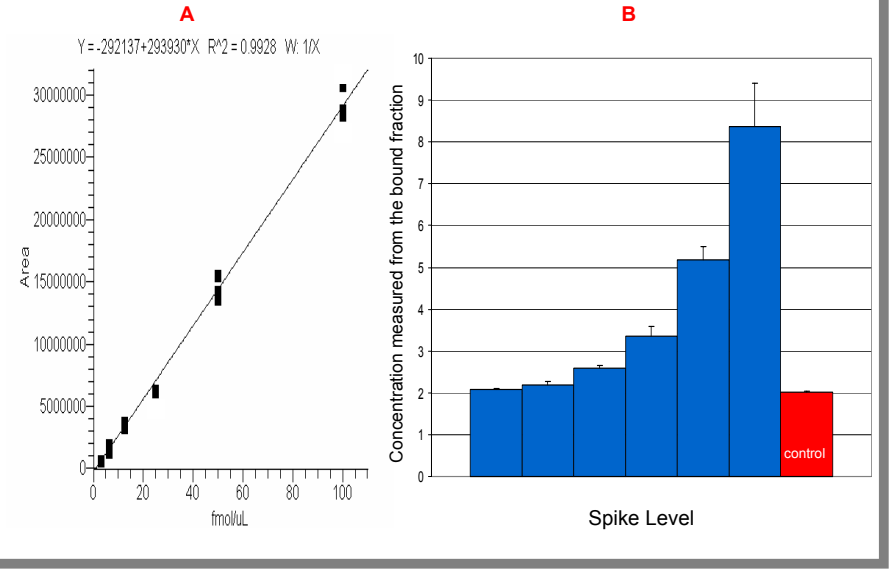
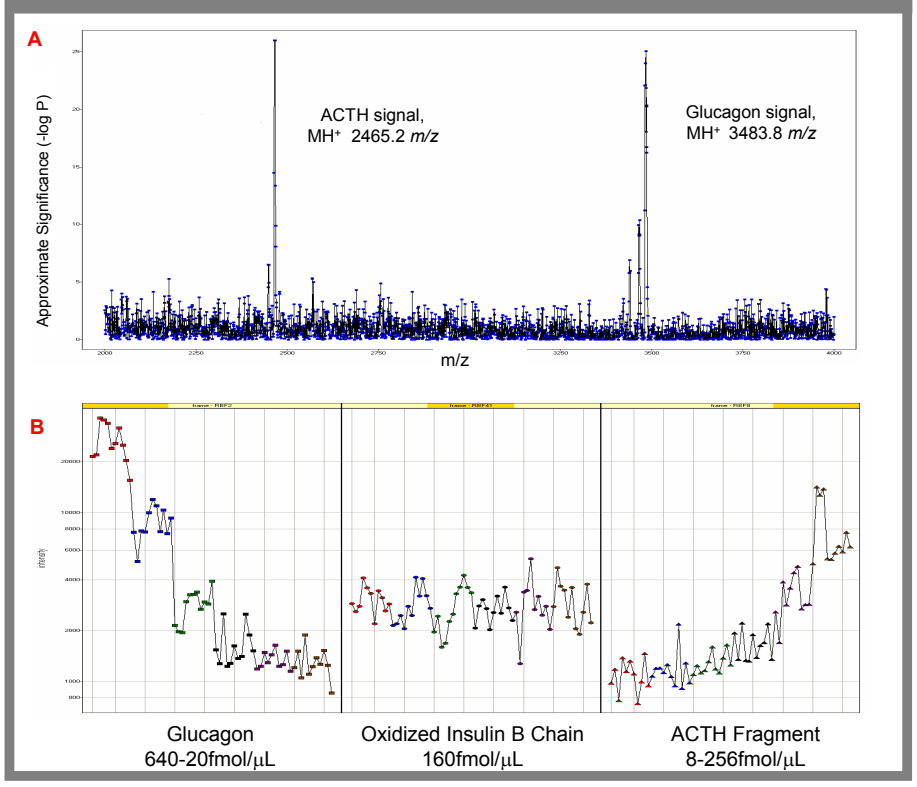


FIGURE 3. (A) Differential analysis of the sixty-six vMALDI spots showing the automatic identification of frames indicating statistically significant changes in relative signal intensity. Values above an intensity threshold triggered framing around 1 *m/z*-wide windows to calculate a p-value for the significance in the change in signal within that frame across the entire sample set. In this example, 66 runs produced 2988 frames (blue squares) in which the two frames scoring the highest significance corresponded to the two peptides spiked at varying levels. (B) Trend analysis of the three spiked peptides across the sixty-six vMALDI spots.



Conclusions

- Automated sample processing of human plasma using hydrophobically-coated magnetic beads with the KingFisher 96 reproducibly enriches spiked and endogenous peptides; the enrichment process preserves the relative abundance information allowing high-throughput differential analysis.
- We are able to accurately identify and detect *from a single scan* statistically significant changes in the response from a set of standard peptides spiked into a complex mixture (bound plasma fraction from magnetic bead enrichment) at levels between 25 and 50 fmol per spot.
- Using this platform we can perform differential and trend analysis to track changes in the levels of individual peptides within a complex mixture.

References

1. Villanueva, Anal. Chem. 2004, 76, 1560-1570

Thermo ELECTRON CORPORATION