A Deeper Look Into Sample Collection and Handling-induced Variability of Plasma Proteins in Proteomics

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Overview

Purpose

Compare plasma peptidome from EDTA vs. BD™ P100 Tubes.

Methods

- Collect blood in EDTA and BD™ P100 Tubes.
- Centrifuge at 2,500*xg* allowing the mechanical separator to isolate the plasma from blood cells.
- Plasma was aliquoted at various time intervals.
- The peptidome was recovered using a 3kDa MW cutoff filter.
- LC-MALDI-MS was employed to analyze the plasma peptidome.
- Virtual 2-D plots were created enabling the direct comparison of EDTA and BD™ P100 plasma peptidomes.

Result

- Successful comparison of plasma peptidome from EDTA vs. BD™ P100 Tubes.
- Presence of protease inhibitors in the BD™ P100 tubes stabilizes both native proteins and peptides.
- Thrombin activity is not exclusive to fibrinogen in blood.

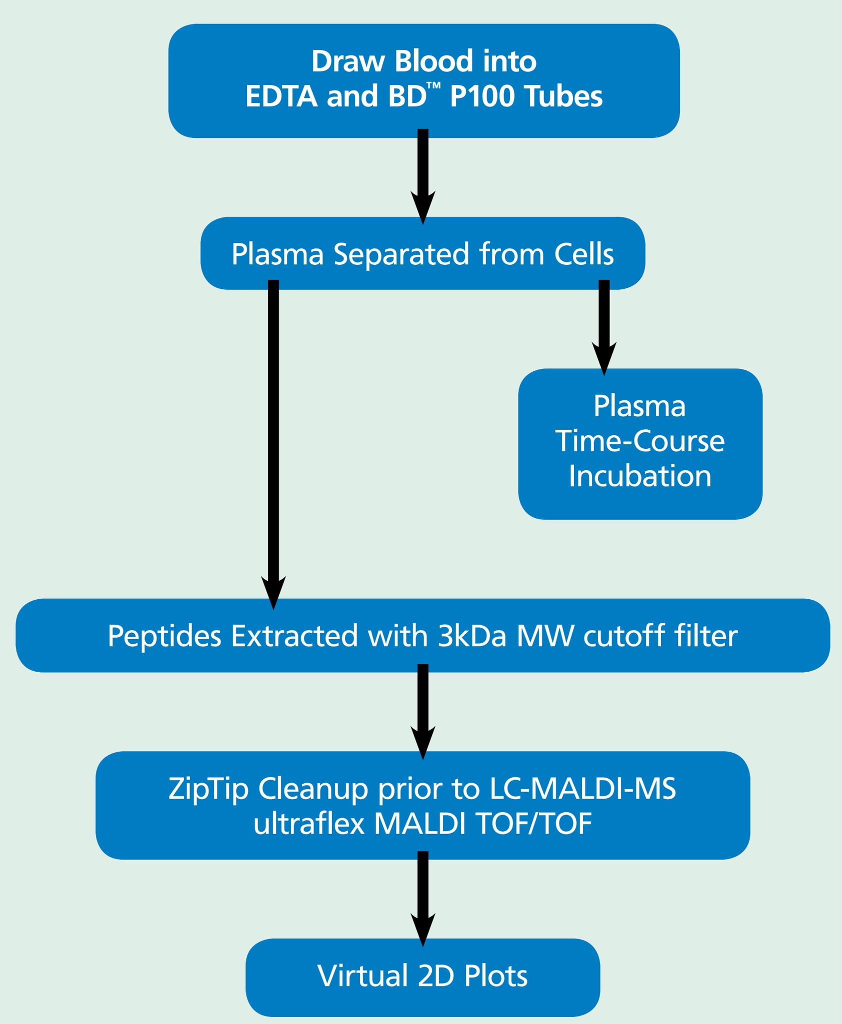
Introduction

The search for proteomic biomarkers from human plasma holds both incredible clinical potential as well as significant challenges.[1] The dynamic range of concentration, known to exceed ten orders of magnitude, is the primary limitations in modern plasma proteomic analyses, typically limited to 10^4–10^6 with current instrumentation. Beyond the well-known dynamic range issues, plasma proteome analysis is further complicated by preanalytical variability, in particular, blood collection and sample handling and processing issues needing to be evaluated. Our previous experiments demonstrated that plasma, collected in evacuated blood tubes including protease inhibitors present at the moment of phlebotomy, yields more time-stable and intact samples.[2] Standard serum and anticoagulated plasma samples, in parallel studies, are measurably less stable. After centrifuging, separating plasma from blood cells, the samples were incubated for different lengths of time, then passed through 3kDa molecular weight (MW) cutoff filters, and the resulting peptides were analyzed by Liquid Chromatography-Matrix-Assisted Laser Desorption/Ionization-Mass Spectrometry (LC-MALDI-MS). MS results indicate "new" peptides being generated *ex vivo* more rapidly in standard EDTA tubes, as compared to measurably increased stability using protease inhibitors (BD™ P100 Tubes*).

In the previous studies, the entire peptide content from each sample was analyzed by direct MALDI-MS. In the current study, we increased the dynamic range of detectable peptides by using reversed-phase chromatography, in a LC-MALDI-MS format. Methodical probing of plasma peptides enables deeper understanding of preanalytical variables associated with sample collection and handling. It further elucidates the beneficial aspect of *in vitro* protease inhibitors and their role in stabilizing plasma proteins acquisition and handling standards. Also, extrinsic thrombin was added to a subfraction of low molecular weight plasma proteins to observe its proteolytical specificity.

**For research use only. Not for use in diagnostic procedures.*

Experimental



BD™ P100 Kit
Blood Collection System for Use in Protein Analysis

Cross-section view of BD™ P100 Plasma Tube* showing immediate, controlled mixing of blood with the PI additive.

**For research use only. Not for use in diagnostic procedures. Patent Pending*

Results

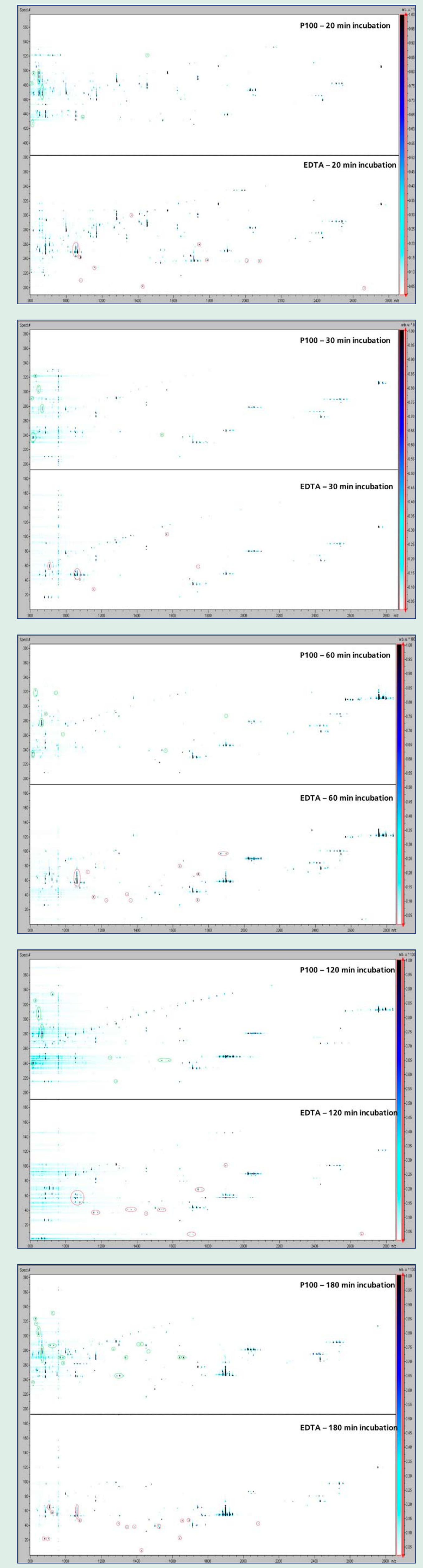
In our previous work, we compared the peptidome from plasma collected in BD™ P100 vs. EDTA tubes (using direct MALDI) as a function of time[2]. Briefly, more new peptides were being produced over time *ex vivo* in EDTA vs. BD™ P100 Tubes, indicating a greater overall protein stability in the BD™ P100 Tubes. However, the number of peptides observed in direct MALDI were limited to a dynamic range of 10^3. To increase the dynamic range analyzed, we combined reversed phase LC with MALDI, which resulted in a greater number of detectable peptides. When comparing the peptidome from BD™ P100 and EDTA tubes directly, the virtual 2-D plots appear complex and complicated (Figure 1). However, a couple of key features stand out.

Several low molecular weight peptides from the BD™ P100 Tube (highlighted in green) remain fairly constant throughout the time-course study. In contrast, this subset of peptides was rarely observed in the EDTA tubes, suggesting early degradation of these peptides in the EDTA tubes.

By contrast, a large number of anomalous peptides can be observed between 20 minutes and 180 minutes for EDTA tubes. With BD™ P100 Tubes, we only start observing this level of deviation after 180 minutes.

A very distinct peptide (1060.5 m/z) dominates in the peptidome from EDTA tubes throughout the entire time-course study, and is only marginally observed (1% compared to the EDTA signal at 20 minutes) in the peptidome from BD™ P100 Tube until the 180 minutes mark. The spectrum for the fraction containing peptide 1060.5 m/z from each time interval is displayed in Figure 2.

Figure 1. Monitoring peptide variance in P100 vs. EDTA over time



Blood was collected from one healthy donor in both BD™ P100 and EDTA tubes and the plasma was isolated by centrifugation. Plasma was sampled at different time intervals and the intrinsic peptidomes were analyzed by LC-MALDI-MS. Figure 1 displays virtual 2-D plots of the peptidome after 20 to 180 minutes of plasma incubation at room temperature. The unique peptides are circled in green (P100) and red (EDTA).

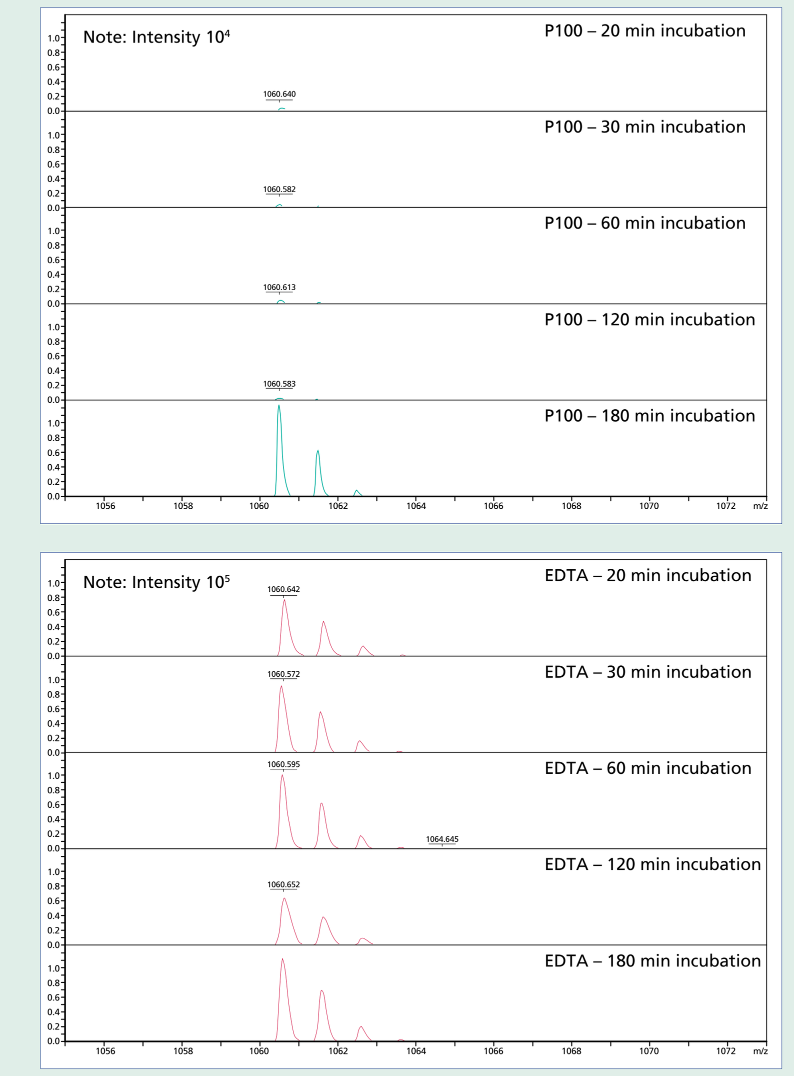
Protein	MASCOT Score	Number of Peptides
Albumin	382	7
Hypothetical Protein Hflu	181	4
Chain D, Apolipoprotein	142	3
Fibrinogen	111	3
Chain B Alpha-1 antitrypsin	96	2

****Note - polymer peaks, with a 44 m/z distribution, present in some graphs (particularly P100 at 120 minutes) are a result of the MW cutoff filter and not the blood collection tubes, based on controlled experiments (data not shown)****

This data convincingly illustrates how peptide 1060.5 m/z is stabilized in the BD™ P100 Tube over the first 2 hours at room temperature. It also suggests this peptide is being produced in the EDTA tube during and after blood collection.

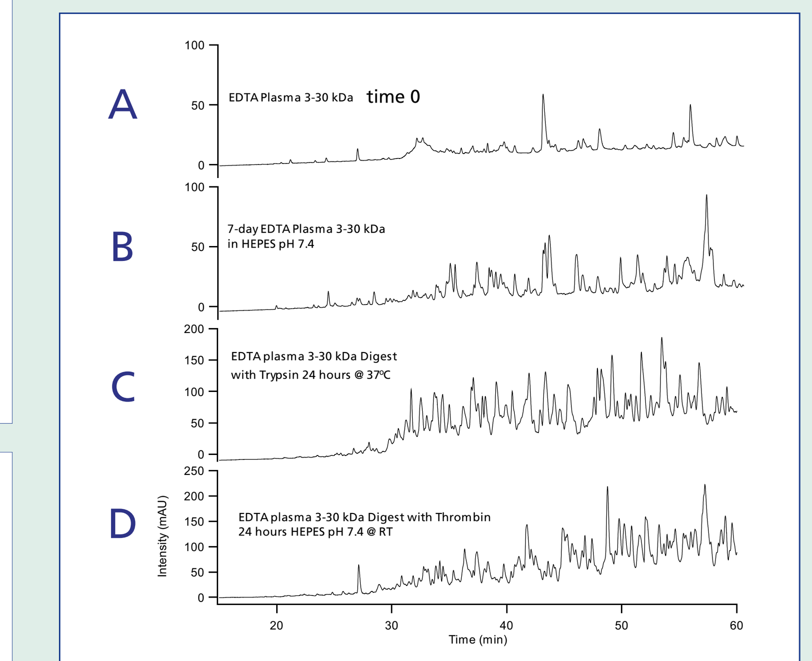
Post Source Decay (PSD) was performed on highly abundant peptides for both peptidomes obtained from EDTA and BD™ P100 using lift mode on the ultraflex (Bruker Daltonics, Germany) for the 20 minute time interval LC-MALDI fractions. Several high abundant proteins, including albumin, apolipoprotein, fibrinogen, antitrypsin, and hypothetical protein Hflu were identified in both the EDTA and BD™ P100 Tubes. However, more peptides from these proteins were identified in the EDTA tube. These peptides contained various different amino acids on their C-terminus, suggesting these are generated by different proteolytic pathways. The combination of results demonstrate a greater protein stabilization beginning at the point of blood collection and over time in the BD™ P100 Tube vs. EDTA. Thus, BD™ P100 displays less peptide variation over time.

Figure 2. Peptide 1060.5 m/z in P100 vs. EDTA



There are many known plasma proteins portraying proteolytic activity, the most notable being thrombin. Thrombin, a serine protease with trypsin-like specificity, is responsible for the degradation of fibrinogen, which produce Fibrinogen Peptide A and B peptides and induces a clot. A controlled experiment was performed in which the low molecular weight plasma proteins were isolated with a 30kDa MW cutoff filter. A 3kDa MW cutoff filter was used to eliminate the presence of any intrinsic peptides. This protein fraction was then incubated with either thrombin at room temperature or trypsin at 37°C. The resulting chromatograms are displayed in Figure 3. Thrombin and trypsin similarly digest plasma proteins. PSD was performed on the highly abundance peptides resulting in the identification of several highly abundance proteins using MASCOT. Each identified peptide contains either an arginine or lysine on the C-terminus resulting from thrombin cleavage, clearly demonstrating thrombin proteolytic activity beyond the "expected" biological function.

Figure 3. Examination of the *in vitro* proteolytic activity of α-thrombin on plasma-derived proteins



The low molecular weight (LMW) proteins (3-30kDa) were removed from plasma (effectively removing fibrinogen), to observe the *in vitro* proteolysis of thrombin on blood proteins without inducing coagulation.

A. Time 0 chromatograph from an RP-HPLC separation of LMW proteome from plasma (EDTA). Some protein/peptide peaks are evident. (24 hours)

B. Time 7 days chromatograph from the same sample in A above showing some intrinsic proteolysis.

C. Time 24 h chromatograph of an aliquot from A above that has been incubated with trypsin. Extensive proteolysis is evident.

D. Time 24 h chromatograph of an aliquot from A above that has been incubated with α-thrombin. Proteolysis similar to trypsin is evident. Peptides observed, following thrombin digestion, in the LC-MALDI-MS all displayed the appropriate R or K C-terminus residues:

Protein	MASCOT Score	Number of Peptides
Proapolipoprotein	664	10
Alpha-1 antitrypsin	201	6
Lipoprotein CIII	180	2

Conclusions

- **Protease inhibitors present in the BD™ P100 Tube increase both protein and endogenous peptide stability starting at the time of blood collection.**
  - **Observation of anomalous peptides between 20 minutes and 180 minutes for EDTA compared to BD™ P100 Tubes demonstrates greater protein stabilization as a result of the protease inhibitors.**
  - **BD™ P100 stabilizes the protein containing peptide 1060 m/z at the point of collection.**
  - **Low molecular weight peptides consistently seen in BD™ P100 Tubes vs. EDTA suggests that protease inhibition protects endogenous peptides in plasma.**
- **Thrombin is not exclusive to fibrinogen degradation. Thrombin cleaves many other blood proteins.**

www.bd.com/proteomics

References:

1. Anderson NL, Polanski M, Pieper R, et al. The human plasma proteome: a nonredundant list developed by combination of four separate sources. *Mol Cell Proteomics.* 2004; 3:311-326.
2. Yi J, Kim C, Finocchio T, Gelfand CA. Preanalytical variability and the stability of human plasma proteins: mass spectrometry changes after blood collection. Presented at 54th ASMS Conference; May 28-June 1, 2006; Seattle, Wash.

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