Intrinsic Preanalytical Variability of Serum Samples is Evidenced in Peptide MALDI MS Spectra

P. O'Mullan, J. Yi, N. Sengupta, and C.A. Gelfand
BD Diagnostics, Franklin Lakes, New Jersey, USA

BD Helping all people live healthy lives

Abstract

Serum is a highly complex sample that contains proteins intrinsic to blood and potentially from various disease states. Some of our recent studies with individual protein analytes have shown that serum is subject to preanalytical variations that can cause detectable variability, complicating the definition of the "true" *in vivo* state of samples. Serum is clotted "in vitro" and centrifuged to remove clotted components, processes that, by definition, could be inducing preanalytical variability on the serum proteome. Changing peptide Mass Spectrometry (MS) patterns should be, and in fact, are easily observed in serum as a function of time.

Thrombin plays a central role in the clotting process and, as a protease, may cause or influence the digestion of other proteins.

This study examines the effects of preanalytical sample handling and thrombin addition on protein stability. Study variables include sample heating to reduce intrinsic protease activity, addition of extrinsic thrombin, and incubation times representing potential clinical scenarios. Samples, examined at specific time points, were quenched; and peptides were extracted, then measured using MALDI-TOF MS. Tandem TOF-TOF was performed for peptide identification, where applicable.

We observed time-dependent patterns of degradation and generation of peptides, in both heated and non-heated serum samples. The addition of thrombin resulted in complete degradation of most peptides initially present, as well as the appearance of multiple "new" smaller peaks across the entire M/z spectra (1000 – 4000 Da). These results suggest that clotting causes the time-dependent variability observed in serum. In addition, intrinsic proteases, such as thrombin, may impart a pronounced *ex-vivo* degradation of blood proteins derived from serum.

Introduction

1. Blood – a tissue that provides a wide array of functions perfusing every other tissue/organ in the body.[1,4]
2. Serum is generally used as an acceptable starting material for many diagnostic assays.[4]
   a. Serum – product of proteolytic clotting process followed by centrifugation to remove solid (clot) materials.
   b. Plasma – the liquid portion of blood. Following collection, cells are spun out to leave the plasma.
3. Clotting process –
   - Proteolytically driven process involving serine proteases creating a cascade effect to form solid fibrin/platelet plugs. [2,4]
   - The time from sample collection to stabilization is extended due to the time needed for complete clotting (~30 minutes) and centrifugation (~10 minutes). This prevents a true "time zero" representation of blood proteins from their "*in vivo*" state.
   - The extended time zero may impact the observed blood proteome and affect the potential discovery of biomarkers.
4. Several factors termed "Preanalytical variables" can affect the observed proteome:
   a. Proteolytic clotting cascade may impact the stability of low abundance proteins.
   b. Time – the time following clotting/centrifugation needs to be examined as circulating proteases may also change the observed proteome.
   c. Heating (pasteurization) – exposure of blood products to heat for several hours to inactivate pathogens. A modified version to reduce intrinsic protease activity is examined here.

Hypothesis

Clotting process affects the suitability of serum for proteome analysis. This is especially relevant when looking for low abundance or labile markers.

- Current diagnostics (e.g. routine serology) rely on characterized, stable biomarkers, but this small "tip of the iceberg" may mask an enormous resource of new potentially labile or low abundance markers.
- Examination of the blood proteome.
  – Dynamic range of proteins in blood ~10^{10} [1,3,6]
  – Stabilization of true "*in vivo*" state of blood proteome appears increasingly essential.
- Serum variability has been demonstrated previously.
  – Quek et al., (2005) have shown a variation in cytokine levels in serum vs. plasma.
  – Baumann et al., (2005) reinforce this idea by demonstrating the variations in relative peak intensities observed within serum, under various conditions, using MS.
- Addition of extra thrombin "*ex vivo*" to a serum sample may serve to amplify potential effects of proteolytic clotting cascade.

Methods

Sample preparation
- Blood was collected from anonymous subjects and serum was processed as follows:
  – Clotted (30-40 minutes)
  – Centrifuged (2500xg for 10 minutes)

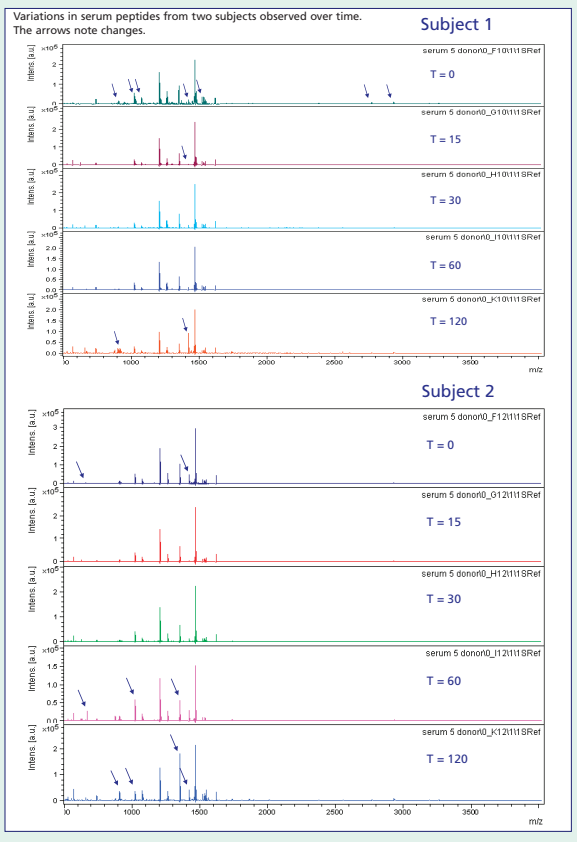
Serum was aliquoted from the clotted tubes into 6 mL Falcon tubes.
Samples were aliquoted into fractions as follows
- Serum control
- Serum + Thrombin (10 U/mL final)
- Serum heated (65°C for 30 minutes)
- Serum heated + Thrombin (10 U/mL final)

Sample treatment
- As outlined above, samples from several donors were collected and aliquoted.
- Aliquots were placed on ice and samples designated for heat treatment were placed at 65°C for 30 minutes in a heat block.
- Following heating, samples were returned to ice to equilibrate.
- Alpha–thrombin was added to appropriate samples at 10 U/mL final.
- Time 0 aliquots were removed and quenched with trifluoroacetic acid (TFA), final concentration of 0.1% v/v, and frozen (-20°C).
- Aliquots were removed at specified time intervals and quenched as described above.

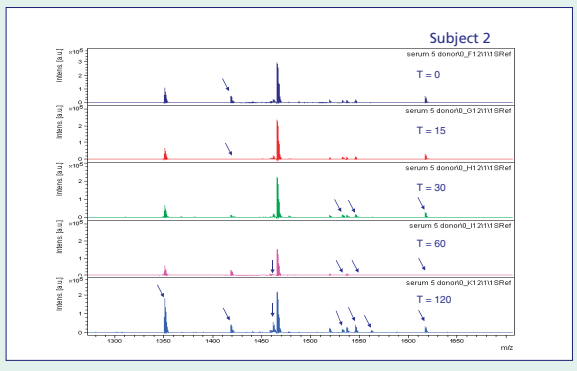
Sample analysis
- Peptides were removed from the quenched samples by ultra filtration through a 10K mw cutoff filter (Millipore) according to the manufacturer's directions.
- Collected peptides were cleaned and processed for MALDI-TOF analysis using ZipTips® (Millipore) according to the manufacturer's directions.
- A saturated solution of alpha-cyano-4-hydroxy-cinnamic acid (about 10 mg/mL) was prepared for the matrix and 1-2 µL was mixed with an equal volume of peptide sample and spotted on a stainless steel target chip from Bruker Daltonics.
- Sample Chips were analyzed using an Ultraflex II MALDI-TOF instrument from Bruker Daltonics.

Results: Serum

- **Time dependent variations in peak intensities are evident in serum samples.**

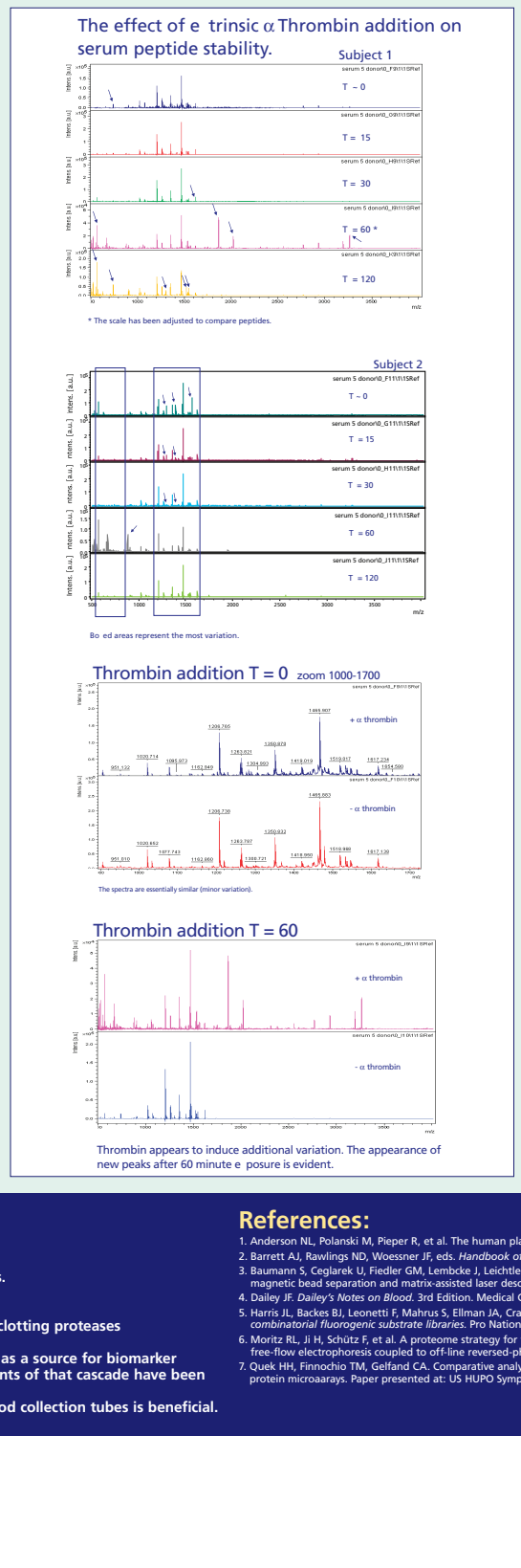


Serum zoom (M/z 1250-1700)



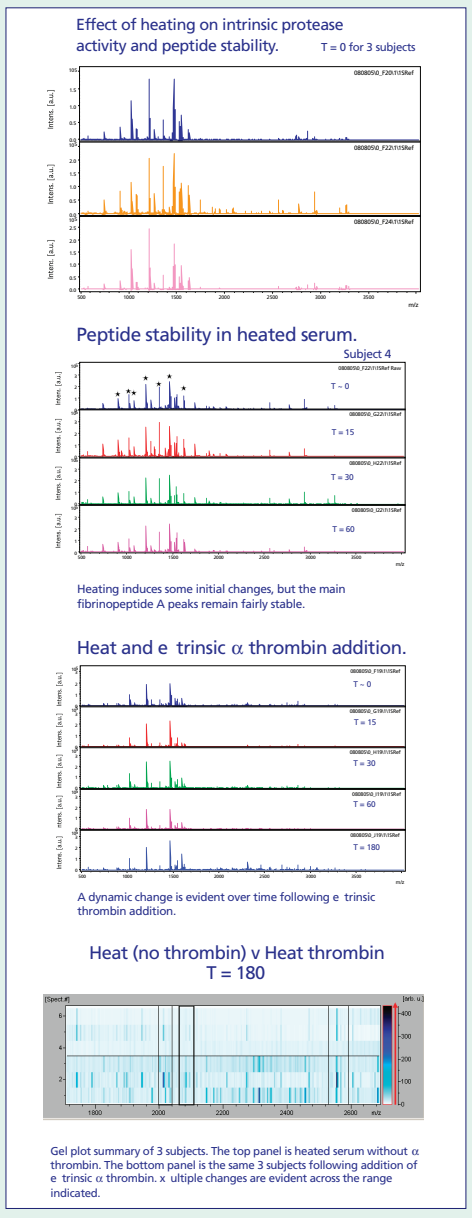
Results: α Thrombin

- **Thrombin-induced variations are evident as early as 15 minutes.**
- **The most obvious effect is seen at 60 minutes (scales are adjusted so the relative peak intensities can be compared).**



Results: Heat and α Thrombin addition

- **Heating samples appears to modify the observed peptides but reduce the variability (evidenced by fibrinopeptide A peaks).**
- **Addition of extrinsic α thrombin to heated samples induces a dynamic change in the peptides observed over time.**



Conclusions

- **Serum appears inherently variable with respect to the observable proteome.**
- **Thrombin (extrinsic) amplifies this effect.**
- **Heating modifies the observable proteome but seems to stabilize the fibrinopeptide A markers.**
  – **Thrombin increases the number of visible peptides across the spectrum.**
  – **The addition of extrinsic thrombin possibly amplifies its effect as a serum protease.**
  – **Serum is the product that results from a proteolytic cascade induced by thrombin, and the clotting proteases remain active in the sample.**
  – **The data suggests that alpha thrombin, directly or indirectly, decreases the utility of serum as a source for biomarker discovery. It initiates a cascade of proteolytic activity. Though specific in function, components of that cascade have been shown to have "promiscuous" activity.[2,5]**
  – **Given the variability in serum, this study suggests that the use of protease inhibitors in blood collection tubes is beneficial.**

References:

1. Anderson NL, Polanski M, Pieper R, et al. The human plasma proteome. *Mol Cell Proteomics.* 2004; 3: 311-326.
2. Barrett AJ, Rawlings ND, Woessner JF, eds. *Handbook of Proteolytic Enzymes,* Vol 1-2. 2nd edition. London, England: Elsevier Academic Press; 2004.
3. Baumann S, Ceglarek U, Fiedler GM, Lembcke J, Leichtle A, Thiery J. Standardized approach to proteome profiling of human serum based on magnetic bead separation and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Clin Chemistry. 2005; 51:6; 973-980.
4. Dailey JF. *Dailey's Notes on Blood.* 3rd Edition. Medical Consulting Group; Arlington, VA: 1996.
5. Harris JL, Backes BJ, Leonetti F, Mahrus S, Ellman JA, Craik CS. *Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries.* Pro National Academy Science. 2000; 97:14; 7754-7759.
6. Moritz RL, Ji H, Schütz F, et al. A proteome strategy for fractionating proteins and peptides using continuous free-flow electrophoresis coupled to off-line reversed-phase high performance liquid chromatography. Anal Chem. 2004; 76: 4811-4824.
7. Quek HH, Finnochio TM, Gelfand CA. Comparative analysis of cytokines in human blood plasma and serum using protein microaarays. Paper presented at: US HUPO Symposium, March 2005; Washington D.C.

www.bd.com/proteomics