

# Inhibition of Intrinsic Proteolytic Activity Moderates Preamanalytical Variability and Stabilizes the Human Plasma Proteome

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## Overview

Human plasma and serum proteins are subject to intrinsic proteolytic degradation both during and after blood collection. By monitoring peptides, we investigated the stability of plasma and serum samples, and the effects of anticoagulants and protease inhibitors on the plasma samples. Serum and plasma were subjected to time-course incubation, and the peptides (750-3200 Da) were extracted and analyzed with MALDI-TOF MS. Peptides of interest were further identified by MALDI TOF/TOF MS. We showed that the mass spectra of plasma and serum are significantly different and the anticoagulants influence the spectra. Furthermore, serum and three commonly-used plasmas show changes in their peptide content as a function of incubation time, suggesting that intrinsic protease and peptidase activities cause the variability and instability of the proteome under *ex vivo* conditions. Immediate mixing of blood samples with protease inhibitors included in an EDTA-plasma collection device, inhibits *ex vivo* variation of sample due to rapid intrinsic proteolysis. The sequence-based results indicate that endoproteases cleave plasma proteins and generate peptides, which are then sequentially truncated by peptidases, including both aminopeptidases and carboxypeptidases. While protected plasma suppresses the generation of bradykinin by inhibition of kallikreins, it also provides an enhancement of complement C3-derived peptides by suppressing the peptidase activity. Overall, our results demonstrate that mixing protease inhibitors immediately with blood during sample collection provides enhanced stabilization of plasma proteome.

## Introduction

The *in vivo* human blood environment contains a diverse range of proteins maintained in a dynamic yet regulated manner. In contrast, the *ex vivo* environment of a collected blood sample is wrought with variability by disruption of pathways, and unregulated proteolysis. Precise control of experimental and analytical processes cannot overcome or otherwise compensate for variations that happen during and after blood collection, and therefore, stabilization of samples starting from blood collection is critical.

Although human plasma proteome research is challenged by its protein complexity, such as a huge dynamic range (1-2), plasma, with its richness of proteins and convenience of sample collection, provides an attractive resource for biomarker discovery. Peptides, as a part of the plasma proteome, have had recent attention due to their potential use for cancer diagnostics (3-6). However, none of protein fragments as biomarkers is either robust enough or has passed validation until very recently (7). There remains the important question whether these peptides are stable enough for a wide clinical application.

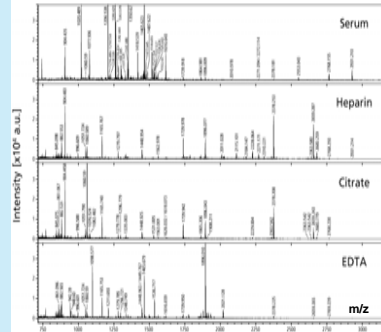
Human serum and plasma contain many active proteases and peptidases (5, 8). To date, instability of serum/plasma proteins and peptides due to intrinsic proteolytic degradation has not been fully or specifically evaluated. In this study, the proteolytic degradation of plasma and serum samples was investigated by peptide analysis using MALDI-TOF MS, including time-course incubation of the samples. We demonstrate that serum and plasma peptides can be dramatically affected by intrinsic proteolysis. The intrinsic proteases are responsible for:

- The peptide differences observed between serum and plasmas;
- The peptide differences among three anti-coagulated plasmas (with EDTA, citrate, or heparin);
- The time-dependent changes of peptides in all samples.

These proteolytic effects are suppressed by the inclusion of protease inhibitors (PIs) in an EDTA-plasma collection device.

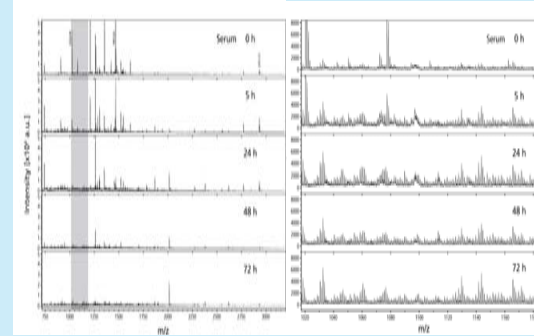
## Results

### Serum versus Plasmas



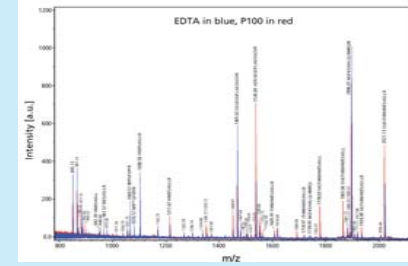
- Serum and plasma were drawn from the same single subject.
- Serum shows significantly different peptides from plasma:
  - More peptides from 2000 to 3000 m/z.
  - Higher abundance of peptides from 1000 to 2000 m/z.
  - The extra peptides are generated due to the *ex vivo* proteolysis during the clotting process (5, 9).
- Heparin plasma displays the most peaks from 2000 to 2500 m/z.
- Citrate and heparin plasmas exhibit more peaks than EDTA plasma from 2000-3000 m/z.

### Serum: time-course MS



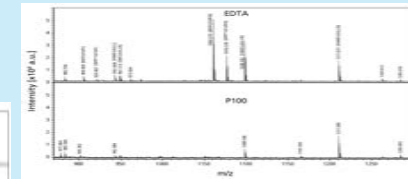
- Time-Course MS indicate the characteristics of a sequential reaction:
  - Some peaks increase in intensity over time, representing the products of on-going proteolytic digestion.
  - Some peaks decrease in intensity with incubation time, representing the substrates of on-going proteolysis.
  - Some peaks increase then decrease over time, representing intermediates in a sequential degradation pathway.
- As a consequence, few peptides are stable over time.
- This suggests that protease and peptidase inhibitors are required to stabilize the serum proteome, and the plasma sample, as shown below.

### Protease Inhibitors: Benefit at "Time 0"



- Two spectra were averaged from ten P100 and ten EDTA samples.
- Identified peptides are shown with sequences on the figure above, and are listed in the table below:
- P100 spectrum shows both more peaks and higher abundance of peptides in 1200-2100 m/z, suggesting stabilization of larger peptides.
- EDTA samples display more peptides in 850-1100 m/z; magnified plot of this range is shown below.

### Magnified EDTA and P100 spectra



- Smaller peptides are less abundant in P100 than in EDTA sample, suggesting that P100 inhibits the generation of these peptides from:
  - their parental proteins (e.g. bradykinin from kininogen proteins), or
  - their parental peptides (e.g. complement C3-derived peptides from C3f).
- P100 protects bradykinin, complement C3- and C4-derived peptides, and fibrinogen peptide A [FPA] (9).

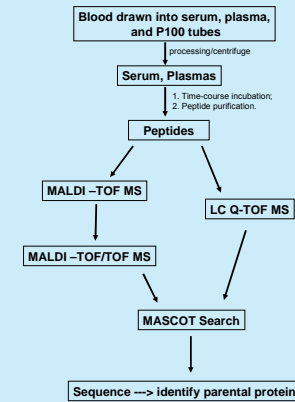
### Table: Identified complement C3-derived peptides and Bradykinins\*

AA sequence	Mass(Da)	MW	Serum	Heparin	Citrate	EDTA	P100
SKKTHRWESASLLR(C3f381-389)	2020.097	2021.128	■	■	■	■	E
SKKTHRWESASLLR	1933.065	1934.080	■	■	■	■	E
ITHRHWESASLLR	1717.934	1718.972	■	■	■	■	E
TRHWESASLLR	1604.850	1605.910	■	■	■	■	E
RHWESASLLR	1210.638	1211.670	■	■	■	■	E
HWESASLLR	1037.922	1038.977	■	■	■	■	R
WESASLLR	960.580	961.570	■	■	■	■	R
SKKTHRWESASLL	1863.996	1865.059	■	■	■	■	E
SKKTHRWESASLL	1776.964	1778.029	■	■	■	■	E
HWESASLL	941.463	942.203	■	■	■	■	R
RPPGSPFR(BK_aa381-389)	1059.561	1060.572	■	■	■	■	R
RPPGSPF	903.460	904.462	■	■	■	■	R
RPP(o)GSPFR	1759.561	1076.57	■	■	■	■	R
RPP(o)GSPF	919.460	920.462	■	■	■	■	R

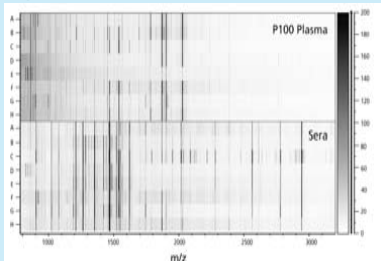
- \*: ■ stands for absence; ■ for presence; ■ for inconclusiveness of presence; E for enhancement; R for reduction.
- Larger C3-derived peptides are enhanced in P100 while smaller peptides are reduced, suggesting that P100 provides a longer life of the primary C3-derived peptides.
- The artificial, *ex vivo* generation of bradykinin is inhibited effectively in P100.

## Experimental Procedures

- Compare peptide spectra of same blood samples collected into different tubes: serum, EDTA, heparin, citrate and BD™ P100™.
- Monitor peptide content by time-course incubation of samples, followed by MALDI-TOF MS (ultraflex II, Bruker-Datascience) analysis.
- Sequence analysis of peptides by TOF/TOF MS (ultraflex II) and LC Q-TOF MS (Waters).
- Monitor peptide variation as an indicator of the instability of the sample proteome.
- Sequences of peptides provide mechanistic information of proteolytic degradation.

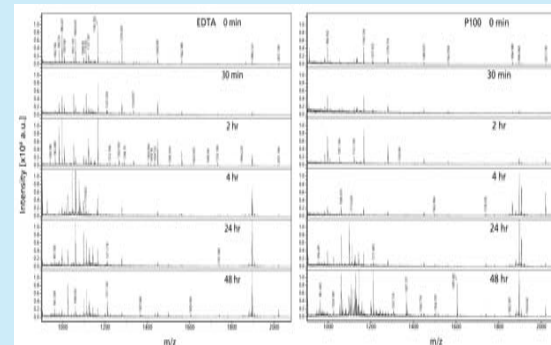


### Protease-Inhibited Plasma versus Unprotected Serum



- Protected plasma samples display lower peaks (1000-1600 m/z) and fewer peptides (2000-3000 m/z), suggesting the stabilization of plasma proteome by the protease inhibitors.
- "Gel view" shows differences of peptide content among the eight individuals tested.
- Demonstrates the reproducibility of the method from sample to spectra.
- Unprotected sera exhibit more and higher abundance peptides, suggesting degradation of the serum samples.

### EDTA Versus P100 Plasmas: Time-Course MS



- EDTA, citrate, and heparin plasmas show similar time-dependent variations (9), suggesting a general instability of human plasma due to intrinsic proteolysis.
- The time-dependent variations of peptide content are extensively moderated in P100 samples.
- P100 sample is reasonably stable for > 2 hours after collection, suggesting improved handling conditions including:
  - Use of protease-inhibited plasma, such as provided by BD P100.
  - Analysis of the sample within a 2-hour timeframe, or
  - Freezing the collected sample within 2 hours.

## Conclusions

- Serum versus Plasmas
  - Most easily detected peptides are *ex vivo*-generated in both serum and plasma, through activation of clotting cascade, complement pathway, and kininogen-kinin contact systems.
  - More peptides are observed in serum than in three commonly used plasmas, implying more degradation in serum and partial inhibition of intrinsic proteolysis by anticoagulants.
  - Intrinsic proteases and peptidases cause sample variability and instability by sequential degradation.
  - Both protease and peptidase inhibitors are required for proteome stabilization.

- P100 Plasma versus EDTA Plasma
  - C3: the sequential degradation is inhibited in P100.
  - C4, FPA: carboxypeptidase activities are suppressed in P100 (9).
  - Bradykinin: its artificial generation is inhibited effectively for > 2 hours in P100.
  - P100 displays fewer number of smaller peptides, and longer life of larger peptides.
- Over all, P100 moderates the proteolytic degradation of proteins and peptides and thus, provides a more robust sample for biomarker discovery, validation, and clinical application.

## References

- Anderson, N. L., Anderson, N. G. *Mol. Cell Proteomics* 2002, 1, 845-867.
- O'Brien, G. S., States, D. J. et al. *Proteomics* 2009, 9, 3226-3245.
- Peterson, E. F., Kuchta, A. M. et al. *Lancet* 2002, 359, 572-577.
- Marshall, J., Kuzochka, P. et al. *J. Proteome Res.* 2003, 2, 361-72 (18).
- Vilanova, J.; Shaffer, D. R. et al. *J. Clin. Invest.* 2006, 116, 271-284.
- Vilanova, J.; Marzocchi, A. J. et al. *Mol. Cell Proteomics* 2006, 5, 1940-52 (32)
- Damaschke, E. P. *J. Proteome Res.* 2006, 5, 2079-82.
- Walsh, P. N. & Ahmad, S. S. *Essays in Biochemistry* 2002, 38, 95-111.
- Yi, J.; Kim, C.; Gelfand, C. A. *J. Proteome Res.* 2006, submitted.