

# Stabilization of the Human Plasma Peptidome by Protease Inhibitors

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## Summary

The proteomics research of human plasma is challenged by its protein complexity and a huge dynamic range (1-2). With its richness of proteins and convenience of sample collection, the plasma, 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). 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 and LC-MALDI-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 (9).

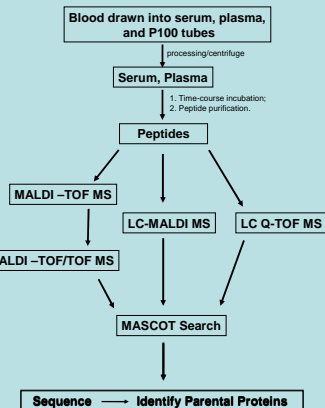
## Experimental Procedures

- 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-Datonic), or LC-MALDI-TOF MS 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.



P100 tube contains:

- A cocktail of protease inhibitors resulting in immediate inhibition during blood collection.
- Coated EDTA as anti-coagulant.
- A mechanical separator\*, which provides a robust barrier between plasma and cells.

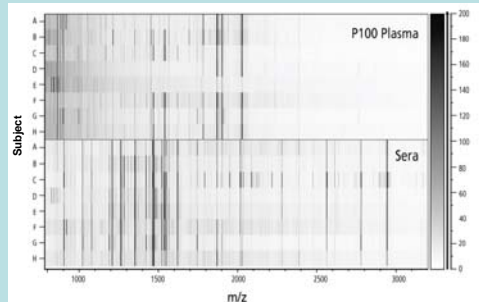


## References

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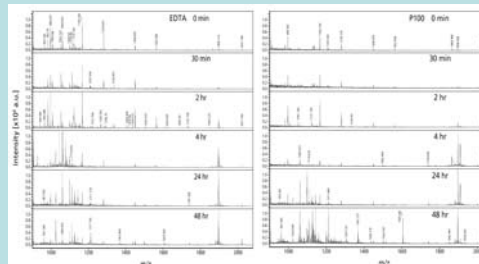
## Results

### Protease-Inhibited Plasma versus Unprotected Serum



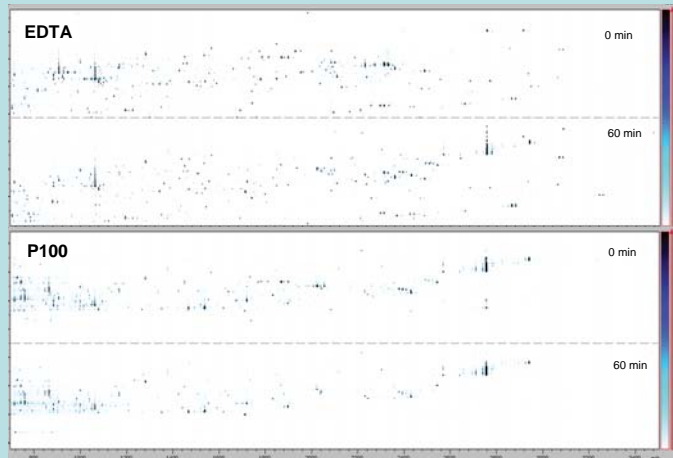
- Protected plasma samples display lower peaks (1000-1600 m/z) and fewer peptides (2000-3000 m/z), suggesting the stabilization of plasma proteome by the protease inhibitors.
- “Gel view” shows differences of peptide content among the eight individuals.
- Demonstrates the reproducibility of the method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

### EDTA versus P100 Plasma: 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.

### Monitoring peptide variation in EDTA and P100 over time with LC-MALDI-MS



Virtual 2D plots of the LC-MALDI-MS data.

- More peptide variations are observed in the EDTA plasma samples over one hour compared to P100 samples, particularly in both 750 – 900 m/z and 2000 – 2500 m/z regions.
- P100 plasma peptidome is more stable over time than the EDTA plasma sample.
- It should also be noted that P100 has less peptides at time 0 and 60 compared to EDTA at its respective time intervals.
- The increase in peptides detected in the EDTA sample is a result of proteolytic activity in the EDTA plasma.
- Therefore, the protease inhibitors in P100 protect the sample both at the moment of blood collection and also over time of sample processing.

## Conclusions

- Serum versus Plasma:
  - Most easily detected peptides are *ex vivo*-generated in both serum and plasma, through activation of clotting cascade, complement pathway, and kinogen-kinins 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, FPs: 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.