

# Proteolytic Activity Quantitation and Protease Inhibition in Human Serum and Plasma Samples for Proteomic Analysis

Angela S. Crawford, Mark D. Schuchard, Richard J. Mehig, William K. Kappel and Graham B.I. Scott

## Abstract

There has been a long standing question as to the level of proteolytic activity naturally occurring in collected human serum and plasma samples. Researchers have commonly added protease inhibitors to serum or plasma samples in order to reduce/arrest proteolytic activity present in such samples. Additions of covalently modifying protease inhibitors (i.e., AEBSF) have been shown to change isoelectric properties, confounding proteomic analysis of such complex samples. In order to better understand if the addition of protease inhibitors is necessary for the study of human serum or plasma, naturally occurring or exogenous protease activity was evaluated using a fluorescent protease detection technology. Protease activity was monitored overnight with or without the addition of proteases in order to challenge endogenous (naturally occurring) protease inhibitors. At a high level, the current study elucidates the effect of endogenous protease inhibitors in regards to the stability of human serum and plasma during sample handling and analysis.

## Introduction

Protease inhibitors are commonly used in proteomic analysis to preserve proteins from endogenous and exogenous proteolytic cleavage. A multitude of protease inhibitors are available which act to inhibit various protease classes, including serine and cysteine proteases, aminopeptidases, acid proteases and metalloproteases. Protease inhibitors are also commonly combined into cocktails for the purpose of concurrently inhibiting several classes of proteases during protein sample preparation and purification activities. Some of these inhibitors are modified peptides and amino acids (e.g., leupeptin, bestatin, E64 and pepstatin A), or native peptides (e.g., aprotinin) and serve as competitive, reversible inhibitors which bind to the active site of proteases but are not cleaved. Other inhibitors [e.g. AEBSF and Phenylmethyl Sulfonyl Fluoride (PMSF)] are competitive, irreversible inhibitors, which covalently attach to important amino acids in the active site of the protease.<sup>1</sup> The use of AEBSF has been shown to negatively impact the analysis of the plasma proteome by 2-DE.<sup>2,3</sup>

Plasma is known to contain a number of endogenous, naturally occurring protease inhibitors including alpha-1 proteinase inhibitor (alpha-1 antitrypsin), antithrombin III, alpha-2 antipain, alpha-1 antichymotrypsin, C1 inhibitor, alpha-2 macroglobulin, inter-alpha trypsin inhibitor, beta-1 anticollagenase and alpha-cysteine protease inhibitor.<sup>3</sup> The effectiveness of these inhibitors for protecting plasma proteins from proteolytic digestion was investigated. A Protease Fluorescent Detection Kit was used to examine the effectiveness of fresh human plasma and serum in inhibiting the activity of trypsin, papain, collagenase and pepsin.

## Methods

### Sample Preparation

#### Serum and Plasma Samples

Blood was drawn into serum and plasma vials. The plasma vials included either citrate, heparin, or EDTA, as anticoagulants. The serum was allowed to clot and the serum and plasma vials were centrifuged for 15 min at 3400 rpm at 5 °C. Additional adjustments to the serum or plasma samples are discussed in the figure legends.

#### HeLa Lysate Samples

HeLa cells were plated at 20,000 cells / mL in 100-cm dishes and allowed to grow for 24 h in Dulbecco's Modified Eagle's Medium (high glucose) (Sigma D5671) supplemented with 5% Fetal Bovine Serum (Sigma F6178) and 4 mM L-glutamine (Sigma G7513). The cells were then lysed using RIPA Buffer (Sigma R0278). The concentration of the cell lysates was adjusted to 1 mg/mL by BCA assay using RIPA buffer.

#### Albumin/IgG Depletion

Plasma samples (50 µL each) were depleted of albumin and IgG with an antibody based depletion resin [ProteoPrep Immunoaffinity Albumin and IgG Depletion Kit (Sigma PROT-IA)] equilibrated with or without 2% (v/v) protease inhibitor cocktail (Sigma P1860), which does not include AEBSF, in equilibration buffer. Protein loads of approximately 200 µg for 2-DE were diluted to 120 µL with water and added to 120 µg of powdered Protein Extraction Reagent Type 4 (Sigma C0356) to produce a final volume of 200 µL in 7 M urea, 2 M thiourea, 1% (w/v) C7BzO, and 40 mM Trizma Base.

#### SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE, 1-DE)

Protein samples were combined with an equal volume of 2 x Laemmli Sample Buffer (Sigma S3401) and heated at 100 °C for 5 min. The protein samples were run on 12 well 4–20% SDS-PAGE gels using Tris-Glycine-SDS running buffer (Sigma T7777) and electrophoresed at 170 V. SigmaMarker™ Wide Range (Sigma M4038) was loaded in the molecular weight marker lane. The gels were stained for 1 h with EZBlue Reagent (Sigma G1041) and destained with water.

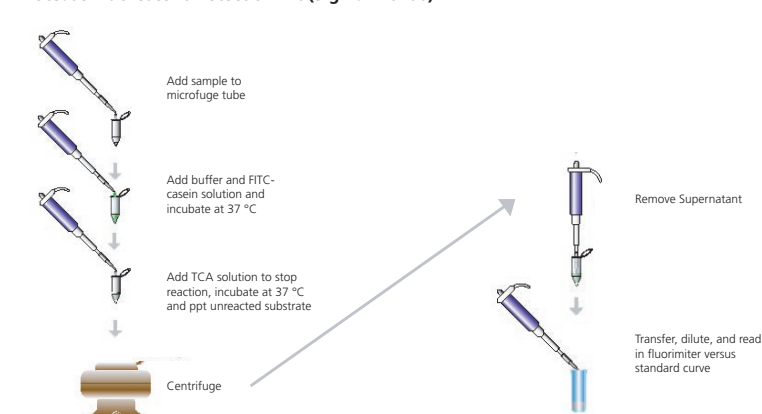
#### Two-Dimensional Electrophoresis (2-DE)

An equivalent volume of normalized depleted plasma was diluted with Protein Extraction Reagent Type 4 and reduced and alkylated using the PROT-RA kit (Tributylphosphine and Iodoacetamide). IPG strips (11 cm, pH 4–7) were rehydrated with the samples and focused overnight (80,000 Vhr). The strips were equilibrated for 15 min with IPG Equilibration Buffer (Sigma I7281) and loaded onto 8–16% SDS-PAGE gels with IPG wells. The gels were electrophoresed at 170V for 1.5 h. The marker lanes contain SigmaMarker Wide Range. The second dimension gel was fixed and stained with EZBlue and then imaged using a Fluor-S™ Multimager (BioRad). The gel images were subsequently analyzed using Phoretix 2D Expression software from Nonlinear Dynamics.

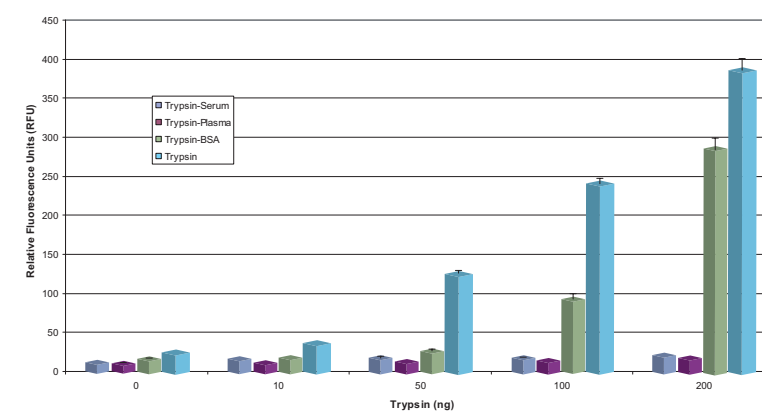
#### Protease Detection Assay

A Protease Fluorescent Detection Kit (Sigma PF0100) was used to examine the inhibition of protease (i.e., trypsin, collagenase, pepsin, and papain) activity by plasma and serum. The reaction mixes (50 µL) consisted of 20 µL of Incubation Buffer, 5 µL of protease and 0–5 µL of either plasma, serum, or BSA (40 mg/mL) or buffer. FITC-casein Substrate (20 µL) was added to each of the tubes and the reactions incubated at 37 °C for 18 h. TCA (150 µL) was added to the tubes to stop the reactions, and the tubes were then incubated at 37 °C for an additional 30 min to enhance precipitation. The vials were then centrifuged for 5 min at 10,000 x g and an aliquot of the supernatant (10 µL) transferred into a 2-mL microcentrifuge tube with 1 mL of Assay Buffer. The fluorescence was measured in a 96-well plate at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a SpectraMax plate reader.

## Protease Fluorescent Detection Kit (Sigma PF0100)



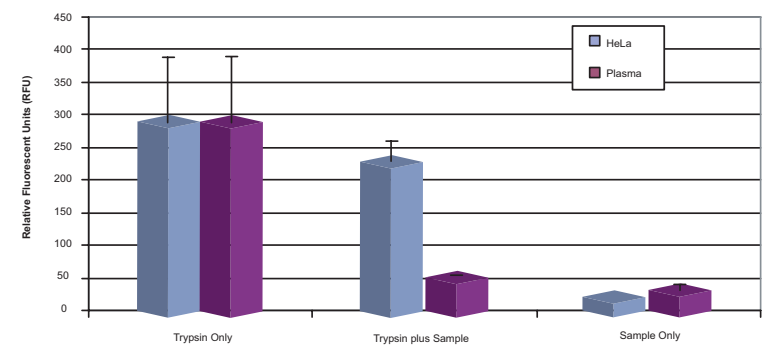
## Results



**Figure 1. Trypsin Inhibition by Citrated Plasma and Serum**

- Citrated plasma and serum (5 µL) inhibited the activity of up to 200 ng of Trypsin.
- A Trypsin-BSA control was added to confirm that the protease inhibition was due to the action of the protease inhibitors and not due to competition of the FITC-casein substrate with Trypsin-digestible protein.
- Additional studies (data not shown) were performed to confirm that the decreased fluorescence produced by serum and citrated, heparinized, and EDTA treated plasma samples was not due to quenching by the samples.
- These results support the inherent inhibition ability of serum and citrated plasma against proteolytic activity.

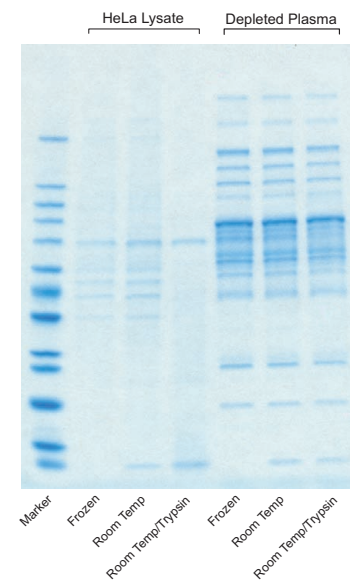
A Protease Fluorescent Detection Kit (Sigma PF0100) was used to examine the inhibition of Trypsin activity by plasma and serum. The reaction mixes consisted of varying amounts of Trypsin (0, 10, 50, 100, and 200 ng) and either plasma, serum, BSA (40 mg/mL) or buffer as described in the Methods section. Each sample was run in duplicate. The relative fluorescence was measured in a 96-well plate. The error bars represent one standard deviation within duplicate samples.



**Figure 2. Citrated Plasma Uniquely Inhibits Trypsin Activity, as Compared to a HeLa Lysate**

- Albumin and IgG depleted citrated plasma shows inhibition of trypsin proteolytic activity (1 µg protease / mL of depleted plasma) whereas the HeLa lysate does not.
- The inherent ability of the plasma to inhibit trypsin activity is unique to the plasma as compared to HeLa lysates.

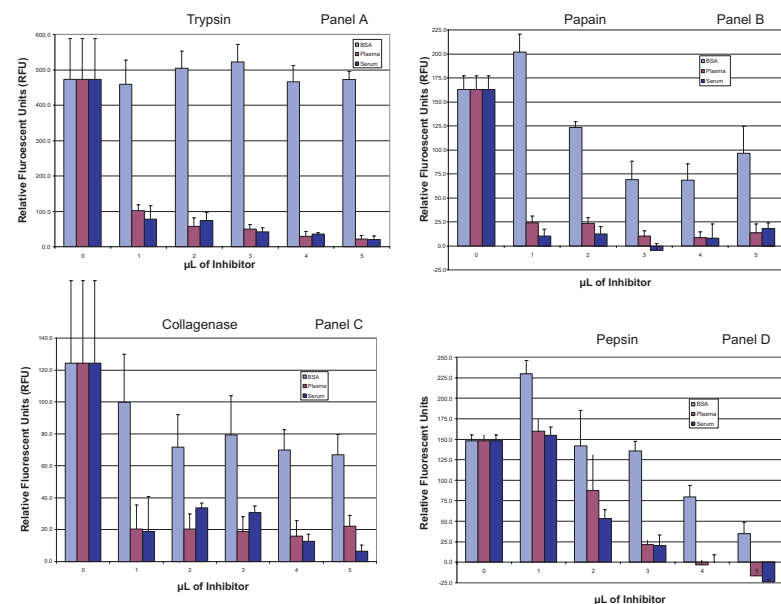
Citrated plasma (50 µL) was depleted of albumin and IgG and the final protein concentration was adjusted to 1 mg/mL by Bradford assay using (PBS) Phosphate Buffer Saline (Sigma P3813). HeLa lysates were adjusted to a concentration of 1 mg/mL as described in the Methods section. The samples were equally split and to half was added trypsin. To the test plasma (100 µL) and test HeLa lysates (100 µL) 0.1 µg of trypsin was added. The samples were allowed to incubate at room temperature for 20 h. Aliquots (10 µL) of each were used to examine the protease inhibition, or lack thereof, using a Protease Fluorescent Detection Kit (Sigma PF0100). Following incubation, the protease detection assay was stopped and measured. As a control for the Protease Fluorescent Detection Kit, 0.1 µg of trypsin was added to 100 µL of Incubation Buffer from the kit. This sample was run alongside the plasma and HeLa lysate samples. Each sample was run in duplicate. The relative fluorescence was measured in a 96-well plate. The error bars represent one standard deviation within duplicate samples.



**Figure 3. Stability of Citrated Plasma, but not HeLa Lysates, When Challenged with Trypsin**

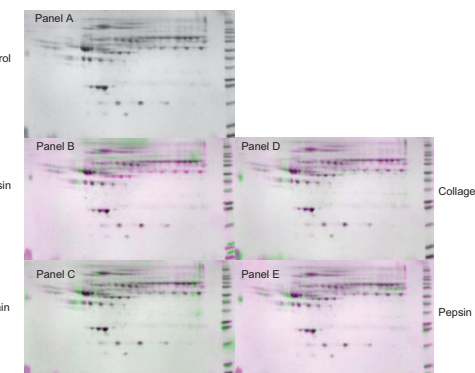
- The protein profiles of the HeLa and plasma samples reinforce the quantitative data (Figure 2), which exhibited degradation of the HeLa lysate proteins and stability of the plasma proteins.
- Additional studies (data not shown) were performed to confirm that with the addition of different protease types (Trypsin, Pepsin, Collagenase, and Papain), similar results were found, in that the HeLa lysate proteins were degraded and the plasma proteins were relatively stable.

Citrated plasma (50 µL) was depleted of albumin and IgG and the final protein concentration was adjusted to a concentration of 1 mg/mL by Bradford assay using (PBS) Phosphate Buffer Saline (Sigma P3813). HeLa lysates were adjusted to a concentration of 1 mg/mL as described in the Methods section. The samples were equally split and to half was added Trypsin. To the test plasma (100 µL) and test HeLa lysates (100 µL) 0.1 µg of trypsin was added. The samples were allowed to incubate at room temperature for 20 hours. Following incubation, a protease inhibitor cocktail (Sigma P1860) was added to the samples at a 1:50 dilution. Aliquots (10 µL) were loaded onto SDS-PAGE gels, as described in the Methods section. As a control, frozen samples of the plasma and HeLa lysates were run.



**Figure 4. Inhibition of Four Proteases by Citrated Plasma and Serum**

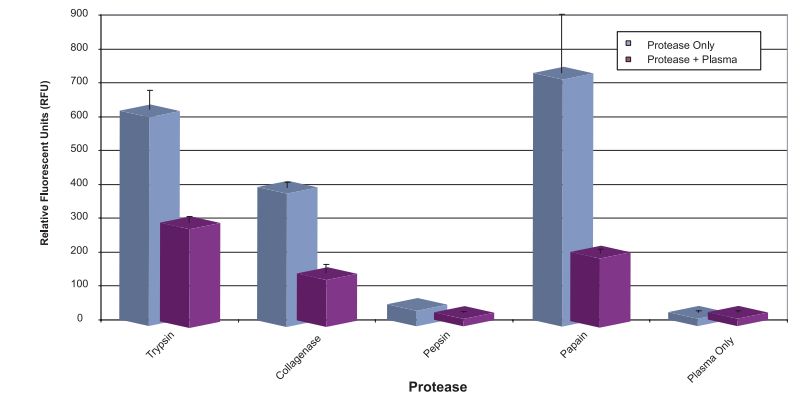
- Citrated plasma and serum (1–5 µL) inhibited the activity of 200 ng of trypsin, papain, and collagenase and the activity of 50 ng of pepsin.
  - A BSA control was added to confirm that the protease inhibition was due to the presence of protease inhibitors and not due to competition of the FITC-casein substrate with digestible protein(s).
  - These results further support the inherent ability of plasma and serum to remain stable against proteolytic activity.
  - Similar results were obtained when using heparinized and EDTA treated plasma (data not shown).
- A Protease Fluorescent Detection Kit (Sigma PF0100) was used to examine the inhibition of protease activity by plasma and serum. The proteases used were as follows: Panel A: Trypsin (200 ng); Panel B: Papain (200 ng); Panel C: Collagenase (200 ng); and Panel D: Pepsin (50 ng). The digestion mixes contained varying volumes of BSA (40 mg/mL) and citrated plasma or serum (0, 1, 2, 3, 4 and 5 µL) as described in the Methods section. The incubation buffer used with each sample was optimized for each protease type. Each sample was run in duplicate. The relative fluorescence was measured in a 96-well plate. The error bars represent one standard deviation within duplicate samples.



**Figure 5. Citrated Plasma Challenged by Four Proteases**

- A number of new protein spots (green) are seen with the addition of the four different proteases (Panels B–E). These protein spots are likely plasma protein fragments cleaved by the proteases. These results are predicted by the Protease Assay (Figure 6).
- The endogenous protease inhibitor, alpha-2 macroglobulin (a2M) disappears (magenta) or is reduced with the addition of the high levels of the different proteases. This is most likely due to inhibition characteristics in which a2M inhibits the four major protease types.<sup>4</sup>
- Intensities of other higher molecular weight proteins, such as ceruloplasmin and fibrinogen, are also greatly reduced, such as with the addition of trypsin (Panel B).

To citrated plasma (100 µL) was added 200 µg/mL of either Trypsin, Papain, Collagenase, Pepsin, or buffer. These digestion mixes were allowed to incubate at room temperature for 20 h. Following incubation, a protease inhibitor cocktail (Sigma P1860) was added to the samples at a 1:50 dilution. Aliquots (50 µL) of each sample were depleted of albumin and IgG and a sample equivalent to 200 ng of depleted plasma, not treated with protease, was added to IPG strip rehydration reagent (Sigma C0356), reduced and alkylated and separated by 2-DE as described in the Methods section. The imaged gels were then analyzed using Phoretix Expression software from Nonlinear Dynamics and the protease treated gels (green spots) displayed as an overlay to the control gel (magenta spots) with no protease added (Panels A–E). Accordingly, the magenta spots on the overlay images (Panels B–E) represent proteins which are at a higher level in the control (Panel A). Similarly, the green spots represent protein (peptides) which are at a higher level in the protease treated samples (Panels B–E). Each sample was run in duplicate. Each gel shown is an averaged gel of the duplicates.



**Figure 6. Four Proteases Inhibited by Citrated Plasma**

- The fluorescent protease assay data mirrors the results observed by 2-DE (Figure 5). To citrated plasma was added either trypsin, papain, collagenase, pepsin, or buffer (200 µg/mL). These digestion mixes were allowed to incubate at room temperature for 20 h. Aliquots (10 µL) of each was used to examine the protease activity, or lack of, using a Protease Fluorescent Detection Kit (Sigma PF0100). The incubation buffer used for the fluorescent protease assay was the supplied buffer in the kit. Following incubation, the protease detection assay was stopped and measured. Each sample was run in duplicate. The relative fluorescence was measured in a 96-well plate. The error bars represent one standard deviation within duplicate samples.

## Conclusions

- Serum and plasma samples appear to be relatively stable against endogenous proteolytic digestion for at least 20 hours, as shown by SDS-PAGE (Figure 3).
- Serum and plasma were most effective at inhibiting trypsin (a serine protease) and also inhibited papain (a cysteine protease), collagenase (a metalloprotease), and to a lesser extent pepsin (an aspartic protease) (Figure 4).
- The inhibition of pepsin is likely due to the presence of alpha-2 macroglobulin, since it is currently the only known aspartic protease inhibitor in human serum and plasma<sup>4</sup> (Figures 4, 5, and 6).
- Proteolytic digestion of the citrated plasma, using high protease levels, reveals the importance of alpha-2 macroglobulin as an endogenous protease inhibitor (Figure 5). Loss of this inhibitor may leave other plasma proteins susceptible to proteolytic degradation.

## References

- Mintz, G. R. *BioPharm.* **1993**, *6*, 34–38.
- Schuchard, M. S. et al. *BioTechniques* (In Press).
- Rai, A. J. et al. *Proteomics* (In Press).
- Travis, J.; Salvesen, G. S. *Ann. Rev. Biochem.* **1983**, *52*, 655–709.