ABRF 2005 Biomolecular Technologies: Discovery to Hypothesis
Poster No. P198-T

Differing Isoform Pattern Changes to Human Plasma Proteins Produced by Covalently Modifying Protease Inhibitors and the Need for Protease Inhibitors

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Abstract

The presence of protease inhibitors in serum or plasma samples has been found to significantly impact the isoform profile of selected plasma proteins as seen on two-dimensional electrophoresis (2-DE) gels. With the inclusion of a protease inhibitor cocktail, many human plasma protein trains (depleted of albumin and IgG) exhibited higher isoelectric point (pI) isoforms. This shift was especially apparent for Apolipoprotein A1 (Apo A1), a relatively high abundance protein. The six protease inhibitor components of the cocktail were individually investigated with albumin and IgG depleted human plasma, and it was shown that the observed effects were caused by 4-(2-Aminoethyl) Benzenesulfonyl Fluoride (AEBSF), a serine protease inhibitor that covalently modifies serine residues. The changes in the pI profile on 2-DE gels have not been previously described. Several serine/tyrosine-containing peptides of Apo A1 were modified with a concomitant mass increase of 183 Da, consistent with the mass increase expected following reaction with AEBSF. These modifications were predominantly seen in the high pI spots. An increase in the number and proportion of modified peptides with increasing pI was also observed. Since plasma contains a number of endogenous protease inhibitors, the need for exogenous protease inhibitors has been investigated. Finally, complementary work was also performed to determine the levels of endogenous proteases in serum and plasma. Specifically, protease concentrations were evaluated using two recently developed fluorescent and colorimetric protease detection technologies.

Introduction

Protease inhibitors are commonly used in proteomic analysis to preserve proteins from endogenous and exogenous proteolytic cleavage. There are a multitude of protease inhibitors available that act upon the various types of protease classes, including serine and cysteine proteases, aminopeptidases, acid proteases, and elastases. Protease inhibitors are 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.[1]

The impact of a protease inhibitor cocktail on the 2-DE profile of human plasma samples was tested. The results showed that several of the visible isoform trains were shifted to a higher pI motif. One component of this protease inhibitor cocktail, AEBSF, was shown to mediate this effect. The mechanism of action of AEBSF is through covalent modification of serine residues in the active site of serine proteases, resulting in the formation of sulfonate esters.[1] AEBSF has also been shown to derivatize other proteins through similar modification of serine and tyrosine residues.[2–5] Such modifications result in molecular weight increases of 183 Da as determined by ESI-MS. Here we illustrate the modification of serine/tyrosine residues in Apolipoprotein A1 isoforms that result in the appearance of higher pI species observed in the 2-DE gels, and their subsequent characterization by mass spectrometry.

Plasma is known to contain a number of natural protease inhibitors including alpha-1 proteinase inhibitor (alpha-1 antitrypsin), antithrombin III, alpha-2 antiplasmin, alpha-1 antichymotrypsin, C1 inhibitor, alpha-2 macroglobulin, inter-alpha trypsin inhibitor, beta-1 anticollagenase, and alpha-cysteine proteinase inhibitor.[6] The effectiveness of these inhibitors for protecting plasma from protease digestion was investigated. A Protease Detection Kit was used to examine the effectiveness of fresh human plasma and serum to inhibit trypsin activity.

Methods

Plasma Protease Inhibition and Albumin/IgG Depletion

A protease inhibitor cocktail (Sigma P8340) or AEBSF (Sigma A8456) were added at a 1% level (1:100 dilution) to human plasma. The protease inhibitor cocktail (100x stock) consists of 104 mM 4-(2-Aminoethyl) Benzenesulfonyl Fluoride (AEBSF), Aprotinin (80 µM), Leupeptin (2 mM), Bestain (4 mM), Pepstatin A (1.5 mM), and E-64 (1.4 mM). The final concentrations in the plasma samples were 1/100th of these values. The individual protease inhibitor AEBSF was dissolved in DMSO at the same concentration as in the cocktail. These 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 1% protease inhibitors, water, or AEBSF in equilibration buffer. Protein loads of 100 or 200 µg for 2-DE were diluted to 130 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.

Two-Dimensional Electrophoresis (2-DE)

A plasma sample and/or an equivalent volume of normalized depleted plasma was diluted with Protein Extraction Reagent Type 4 and reduced and alkylated using PROT-RA (Tributylphosphine and Iodoacetamide). IPG strips (11 cm, pH 4–7) were rehydrated with the samples and focused overnight (85,000 Vhr). The strips were equilibrated for 15 min with IPG Equilibration Buffer (Product Code I 7281) and loaded onto SDS-PAGE gels with IPG wells. The gels were electrophoresed at 170V for 1.5 hours. The marker lanes contain SigmaMarker Wide Range. The second dimension gel was fixed and stained with EZBlue (Sigma G1041).

Gel Analysis and In-Gel Digestion

Stained gels were imaged using a Fluor-S™ MultiImager (BioRad) and evaluated using the Phoretix Expression 2-DE imaging software from Nonlinear Dynamics. Spots of interest were manually cut from the gel and the proteins tryptically digested overnight at 37 °C using the Trypsin Profile IGD Kit (Sigma PP 0100). The extracted peptide digests were dried at 30 °C using a vacuum centrifuge.

Protein Identification by MALDI-TOF Mass Spectrometry

Tryptic peptides derived from each spot were submitted for identification by MALDI-TOF peptide mass fingerprinting and post source decay (PSD). Dried samples were reconstituted in 0.1% Trifluoroacetic acid (TFA) (typically 10–20 µL), mixed 1:1 with matrix solution (α-cyano-4-hydroxycinnamic acid, 5 or 10 mg/mL in 70% acetonitrile, 0.03% TFA). Aliquots (1 µL) were spotted on a MALDI target and allowed to dry at room temperature before introduction into the mass spectrometer. Mass spectra were acquired in positive ion reflectron mode after close-external calibration using Bradykinin 1–7 and Insulin Oxidized B-chain as standards. Spectra were summed over approximately 100 shots, using a Shimadzu-Biotech Axima CFR*plus* instrument. PSD mass spectra were acquired by the summation of approximately 200–500 shots, with an appropriate ion gate setting for m/z discrimination. Fragmentation was augmented by collision-induced dissociation (CID) using air as the collision gas. Protein identification was performed using MASCOT database searching at http://www.matrixscience.com with the appropriate search engine depending on data type (Peptide Mass Fingerprint or Sequence Query for PSD data). Search parameters were typically restricted to *homo sapiens* taxonomy for use of the NCBInr database. Enzyme selection was Trypsin, with either 1 or 2 missed cleavages permitted. Carbamidomethylation of cysteines was selected as a fixed modification. Protein mass was unrestricted and peptide mass tolerance typically set at 250 ppm. Mass values were entered as monoisotopic MH+. In addition, for Sequence Query entries the peptide charge was selected as 1+ and the peptide and MS/MS tolerances set at 250 ppm and 2 Da respectively. Monoisotopic mass values were used and the instrument selection was MALDI-TOF-PSD.

Protease Detection Assay

Blood was drawn into serum and citrated plasma vials. The serum was allowed to clot and both serum and plasma vials were centrifuged. A Protease Fluorescent Detection Kit (Sigma PF 0100) was used to examine the inhibition of trypsin activity by plasma and serum. The reaction mixes (50 µL) consisted of 20 µL of Incubation Buffer, 5 µL of varying amounts of trypsin and 5 µL of either plasma, serum, 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 4.5 hrs. 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 centrifuged for 5 minutes at 10,000 *x* g. An aliquot of the supernatant (10 µL) was 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 the emission wavelength of 535 nm using the SpectraMax plate reader (Molecular Devices).

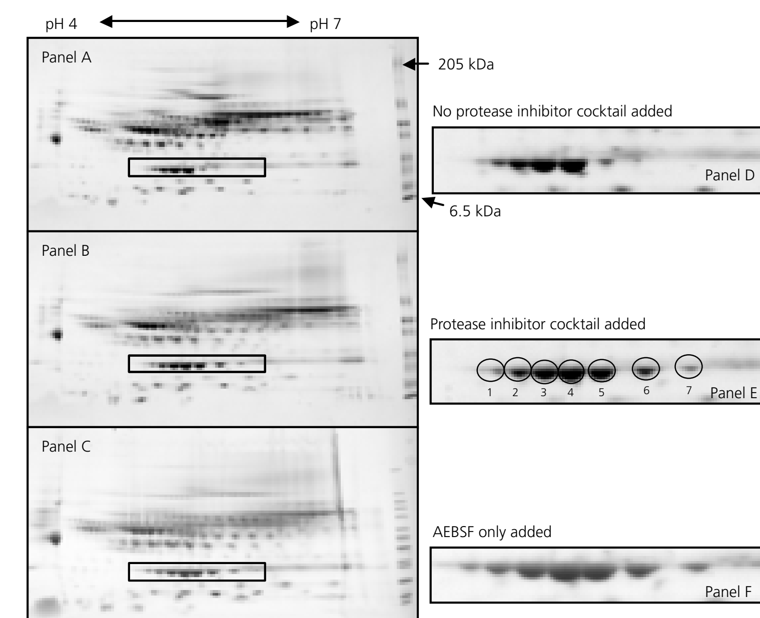

Figure 1: Protease Inhibitor Cocktail and AEBSF Changes the Isoform Profile of Apo A1 in Citrated Plasma

- Seven spots were all identified as Apolipoprotein A1 (ApoA1) with high confidence (MOWSE score greater than 76) and high sequence coverage (greater than 43%).
- The intensity of the two high pI isoforms have been substantially increased with the inclusion of the protease inhibitor cocktail. Conversely, the intensity of the lower pI isoform spots decrease with the addition of the protease inhibitors.
- Specifically, the intensity of the higher pI forms in the Apo A1 protein train only increased when AEBSF was added in isolation from the other constituents of the protease inhibitor cocktail (Panels C and F).

Three samples (50 µL) of citrated plasma were subjected to the addition of either water (Panel A), protease inhibitor cocktail (Panel B), or AEBSF (Panel C). These three samples were each depleted of albumin and IgG. The depleted samples (100 µg for Panels A and B, and 200 µg for Panel C) were separated on 2-DE gels as described in the Methods section using 4–20% gels for the second dimension. Panels D, E, and F are magnifications of the Apo A1 train from gels A, B, and C, respectively. Seven spots were cut from the gel in Panel E and in-gel digested. The seven protein spots were identified by MALDI-TOF MS.

1	RHFWQQDEPP QSPWDRVKDLATVYVDVLKDSGR**<u>DYVSQFEGSALGK</u>**QLNL
51	KLLDNWDSVT STFSKLREQLGPVTQEFWDNLEKETEGLRQEMSKDLEEVK
101	AK**<u>VQPYLDDFQK</u>**KWQEEMELYRQKVEPLRAELQEGARQKL HELQEKLSPL
151	GEEMRDRARAHVDALR**<u>THLA PYSDELR</u>**QRLAARLEALKENGGARLAEYHA
201	KATEHLSTLSEKAKPALEDLRQGLLPVLESFK**<u>VSFLSALEEYTK</u>**KLNTQ

Figure 2: Four of the Tryptic Peptides Derived from ApoA1 Were Identified as Having Partner Peptides with a +183 Da Adduct

These four peptides are delineated via the combination of bold text and underscoring in the amino acid sequence of Apo A1. The peptides having a +183 Da adduct were confirmed by PSD (data not shown).

Figure 3a

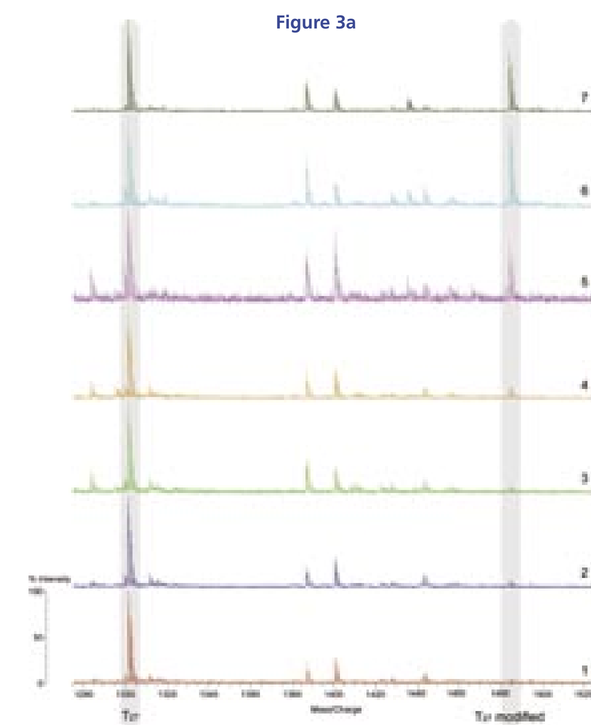

Figure 3b

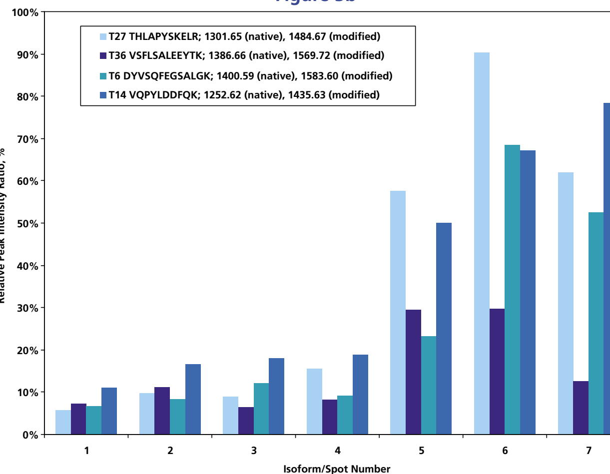

Figure 3: Higher pI Isoforms of Four Apo A1 Peptides Have Increased Levels of the +183 Da Modifications

- The relative abundance of one pair of partner peptides (i.e., 1301 and 1484 Da) was compared (Figure 3a) across the isoform train spot sequence.
- The four modified peptides have a markedly lower abundance in isoforms 1–4, relative to elevated levels in isoforms 5–7 (Figure 3b).

Positive ion reflectron MALDI-TOF mass spectra are shown in the m/z range of 1275–1525 for Apolipoprotein A-I isoforms 1–7 (low to high pI, respectively). All spectra are normalized to 100% of the unmodified peak intensity. The relative peak intensity ratio is calculated by dividing the actual mV signal for the modified peak by the actual mV signal for the unmodified peak, expressed as a percentage.

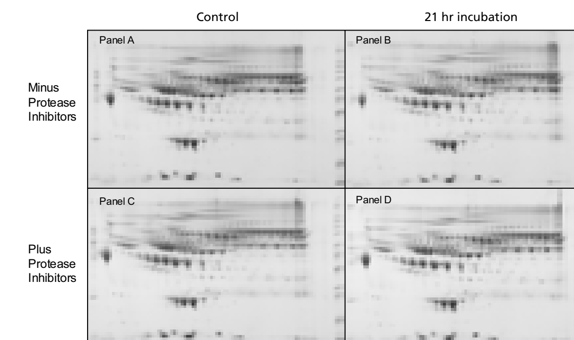

Figure 4: Citrated Plasma Shows No Apparent Protein Degradation on the 2-DE Gels Following a 21 hr Room Temperature Incubation

- The protein profiles of the control (immediately frozen) and the test sample (21 hr incubation) were essentially identical.
- The addition of protease inhibitors did not affect the profile.
- The normalized volume of a number of protein spots on the gels were compared and no statistically significant change was evident (data not shown).

A fresh vial of citrated blood was drawn and centrifuged. The plasma was split and to half of the sample protease inhibitors were added (without AEBSF, Sigma P1860). Gels in Panels A and B were generated from plasma without protease inhibitors and Panels C and D were generated with protease inhibitors. Aliquots were immediately frozen at –70 °C (Controls, Panels A and C). Aliquots were allowed to incubate at room temperature for 21 hours (Panels B and D). Aliquots (50 µL) of each were depleted of albumin and IgG and 200 µg were added to IPG strip rehydration reagent (Sigma C0356) and separated by 2-DE as described in the Methods section. The second dimension gels used were 12.5% (w/v) acrylamide. The gels were stained, imaged, and analyzed using Phoretix Expression software from Nonlinear.

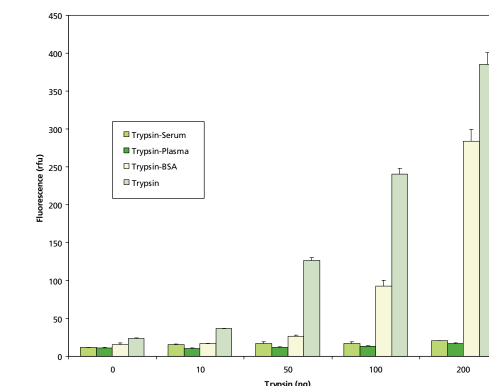

Figure 5: Citrated Plasma and Serum (5 µL) inhibited the Activity of up to 200 ng of Trypsin

- A Trypsin-BSA control was added to confirm that the protease inhibition was attributed to the action of protease inhibitors and not due to competition of the FITC-Casein substrate with Trypsin-digestible protein.
- These results further support the inherent ability of serum and citrated plasma to remain stable against proteolytic activity.

A Protease Fluorescent Detection Kit (Sigma PF 0100) 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, 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.

Conclusions

- Inclusion of AEBSF in a plasma sample increases the number of higher isoforms of Apo-A1 as seen by 2-DE and reduces the overall gel resolution.
- The covalent modification of serine/tyrosine residues by AEBSF adds a positively charged amine group, which shifts the proteins to higher pI forms and generates artifactual isoform spots in protein trains. Therefore, inhibitors such as AEBSF should be used judiciously.
- The citrated plasma protein profile appears to be relatively stable against proteolytic digestion for 21 hrs as seen on 2-DE gels. For this specific plasma sample type, inclusion of protease inhibitors does not appear to be necessary if two 2-DE is employed as the workflow strategy.
- The use of the Protease Detection Kit further illustrates the ability of endogenous protease inhibitors in human plasma (and serum) to inhibit proteolytic activity, which is likely the reason for the apparent citrated plasma stability.

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01902-021104