

The Protease Inhibitor 4-(2-Aminoethyl) Benzenesulfonyl Fluoride Covalently Modifies Human Plasma Proteins and Changes Protein Isoform Profiles

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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 which covalently modifies serine residues. Several serine-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. A model is proposed for the random or stochastic coupling of AEBSF derived moieties to serine residues throughout Apo A1 and potentially other plasma proteins.

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 which act upon the various types of protease classes, including serine proteases, 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 PMSF) are competitive, irreversible inhibitors, which covalently attach to important amino acids in the active site of the protease.

This study reports the outcome of recent investigations into the effects of inclusion or omission of protease inhibitors on depleted plasma samples, via a "routine" proteomic approach to protein characterization. Subsequent analysis of these samples by 2-DE 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 function of the inhibitor is through covalent modification of serine residues in the active site of serine proteases, resulting in the formation of sulfonate esters¹. AEBSF has also been shown to derivatize other proteins through similar modification of serine residues². Such modifications result in molecular weight increases of 183 Da as determined by ESI-MS. In this paper we illustrate the modification of serine 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.

Methods

Plasma Protease Inhibition and Albumin/IgG Depletion

Plasma samples (HUPO PPP No. BDAA01-Cit or BDAA01-Hep) were thawed and either water, protease inhibitor cocktail (Sigma P 8340), or AEBSF (Sigma A 8456) added at a 1% (v/v) level (1:100 dilution). The protease inhibitor cocktail (100x stock) consists of 104 mM 4-(2-Aminoethyl) Benzenesulfonyl Fluoride (AEBSF), Aprotinin (80 µM), Leupeptin (2 mM), Bestatin (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% (v/v) 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 C 0356) 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 (2DE)

The plasma samples were reduced and alkylated using PROT-RA (Tributylphosphine and iodoacetamide). IPG strips (Product Code I 3531, 11 cm, pH 4-7) were rehydrated with the samples and focused overnight (60,000 Vhr). The strips were equilibrated for 15 min with IPG Equilibration Buffer (Product Code I 7281) and loaded onto 4-20% 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. The gels were imaged using a Fluor-S™ Multimager (BioRad).

Western Blotting

Gels for Western blotting immunodetection were incubated in 1x BSN [48 mM Tris, 39 mM glycine, 0.00375% (w/v) SDS, 20% methanol, pH 9.2] for 20 minutes. The proteins in the gels were semidry transferred to PVDF membrane (Immobilon P, 0.45 µm) at 12 V for 40 min. The blots were then stained with Ponceau S (Sigma P 7170) for 5 min and washed with water to confirm protein transfer. The Ponceau S staining was completely stripped with PBS-Tween 20 (Sigma P 3563) for 30 min and the membrane blocked with PBS-5% milk (Sigma P 4739) for 30 min. Primary antibody incubation was carried out with 7.5 µL of IgY anti-ApoA1 (Genway) in 15 mL of PBS-5% milk (1:2000 dilution) for 90 min at room temperature. The blots were washed twice for 5 min each with PBS-Tween 20 and then incubated with 1.5 µL of anti IgY-HRP conjugate secondary antibody (Genway) in 24 mL of PBS-5% milk (1:16000 dilution) for 60 min at room temperature. The blots were finally washed three times for 5 min each with PBS-Tween 20 and developed with Tetramethylbenzidine (TMB) substrate (Sigma T 0565) for one minute.

Gel Analysis and In-Gel Digestion

Stained gels were imaged 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 typically 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 mass fingerprinting and post source decay (PSD). Dried samples were reconstituted in 0.1% 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+ 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 NCBI nr 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 400 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 400 ppm and 2 Da respectively. Monoisotopic mass values were used, and the instrument selection was MALDI-TOF-PSD.

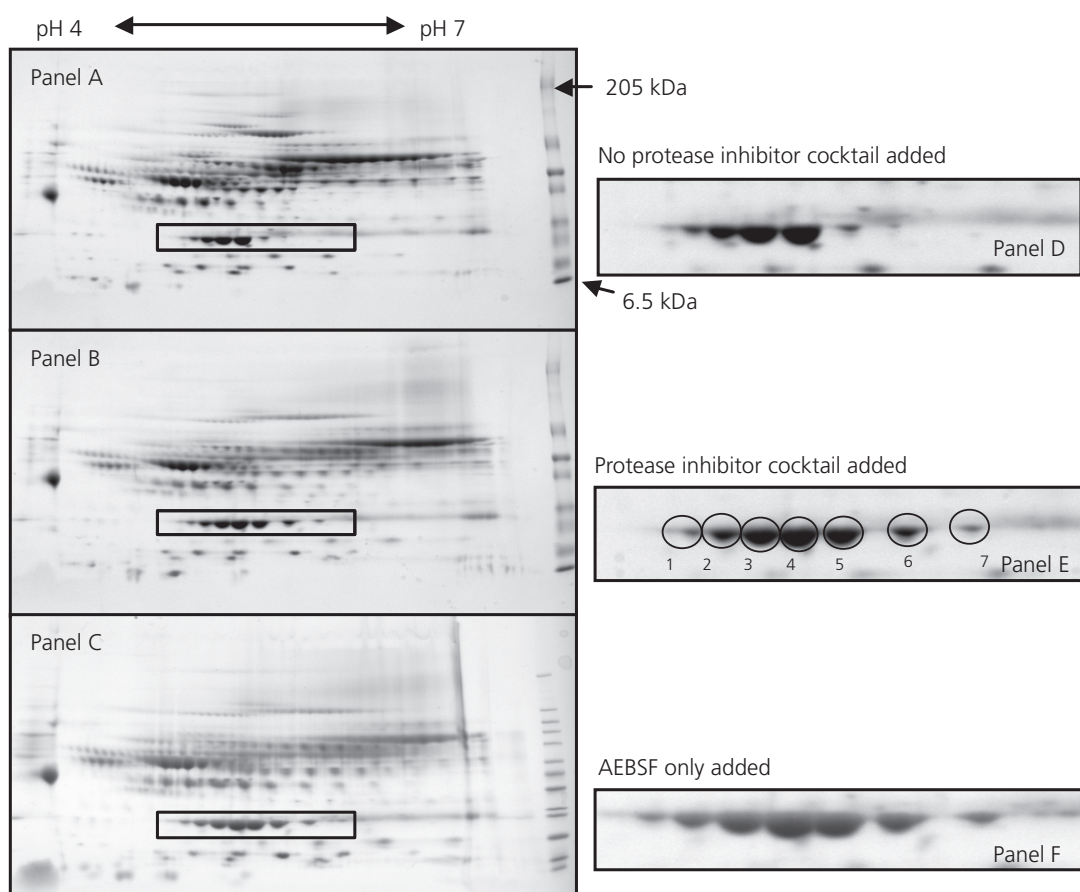


Figure 1: Impact of a Protease Inhibitor Cocktail on the 2DE Pattern of Human Plasma

- The intensity of the two high pI isoforms have been substantially increased with the inclusion of the protease inhibitor cocktail.
- The non-inhibited sample (Panels A and D) shows significantly fewer higher pI isoforms as compared to the samples with inhibitors present (Panels B, C, E and F).
- The intensity of the lower pI isoform spots decrease with the addition of the protease inhibitors having a concomitant increase of the higher pI isoforms (data not shown).
- 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 (HUPO PPP BDAA01-Cit) 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. Panels D, E, and F are magnifications of the Apo A1 train from gels A, B, and C respectively.

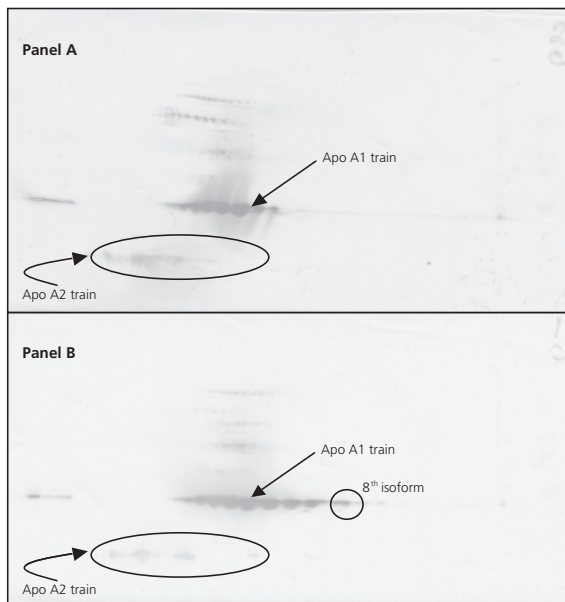


Figure 2: Identification of Apolipoprotein A1 Isoforms by Western Blotting

- Isoforms 1-4 are seen in high abundance both without (Panel A) or with (Panel B) the protease inhibitor cocktail.
- The relative amounts of isoforms 5-7 are significantly higher with the inhibitor cocktail and isoform 7 is essentially not seen in the absence of the cocktail.
- All 7 spots were identified by MALDI with high confidence (MOWSE score greater than 148) and high sequence coverage (greater than 59%). (Data not shown.)
- An 8th isoform (highest pI), detected via Western blotting, was not detected on a Coomassie stained gel.
- Other proteins were detected via interrogation with the anti-Apo A1 antibody, such as the lower molecular weight train highlighted in Figure 2. The spots in this train have molecular masses and pI's similar to Apolipoprotein A2.
- Higher molecular weight trains were observed in Figure 2 but were not identified and consequently may form the basis for future investigation.

Two samples (50 µL) of citrated plasma (HUPO PPP BDAA01-Cit) were subjected to the addition of either water (Panel A), or protease inhibitor cocktail (Panel B). Both samples were each depleted of albumin and IgG. The depleted samples (100 µg) were separated on 2-DE gels then transferred to PVDF membranes as described in the Methods section. Both blots were subjected to analysis by Western blotting using an anti Apo A1 antibody, and colorimetrically detected using TMB substrate.

1 RHFVQDDEPP QSPWDRVKDLATVYVDVLKDSGRDYSVQFEGSALGKQLNL
51 **KLLDNWDSVT STFSK**LRQLREQLGPTQEFWDNLEKTEGLRQEMSKDLEEVK
101 AKVQPYLDDF QKQVQEEEMELYRQKVEPLRAELQEGARQKL HELQKLSPL
151 GEEMDRARAHVDALR**THLA PYSDEL**RQLAARLEALKENGARLAEYHA
201 KATEHLSTLSEKAPALDLRQGLLPVLEFSK**VSFLSALEEYTK**LNLTQ

Figure 3: Amino Acid Sequence of Apo A1

The four tryptic peptides which have identified partners are delineated via the combination of bold text and underscoring, in the amino acid sequence of Apo A1.

Peaks	m/z		Spot Number							Peptide Sequence	
	Expected	Actual	1	2	3	4	5	6	7		
T ₂₇	1301.52	1301.65	+	+	+	+	+	+	+	+	THLAPYSDELRL
T ₂₇ mod	1484.68	1484.67	+	+	+	+	+	+	+	+	THLAPYSDELRL
T ₃₆	1386.72	1386.66	+	+	+	+	+	+	+	+	VSFLSALEEYTK
T ₃₆ mod	1569.75	1569.72						+	+	+	VSFLSALEEYTK
T ₆	1400.67	1400.59	+	+	+	+	+	+	+	+	DYVSQFEGSALGK
T ₆ mod	1583.7	1583.6						+	+	+	YVSQFEGSALGK
T ₈	1612.79	1612.7	+	+	+	+	+	+	+	+	LLDNWDSVTSTFSK
T ₈ mod	1795.82	1797.79							+	+	LLDNWDSVTSTFSK

Table 1: Comparative Tryptic Peptide Lists for Each Isoform

This table details the tryptic peptides observed in MALDI-TOF mass spectra, and partner peptides at Δm/z = +183 Da, corresponding to sulfonate ester formation via the reaction with AEBSF. Note the presence of serine residues in peptide sequences and the appearance of modified peptides in higher pI spots, consistent with the incorporation of additional charged species following reaction with AEBSF. A "+" denotes the observation of the indicated species in the mass spectra.

The characterization of the Apo A1 isoform train was accomplished by MALDI-TOF MS evaluation of peptides derived from each of the 7 spots (Table 1). Four of the nine Apo A1 peptides identified were found to have a partner peptide with a molecular weight increase of 183 Da. The four partner peptides were identified from the high pI isoforms (5-7).

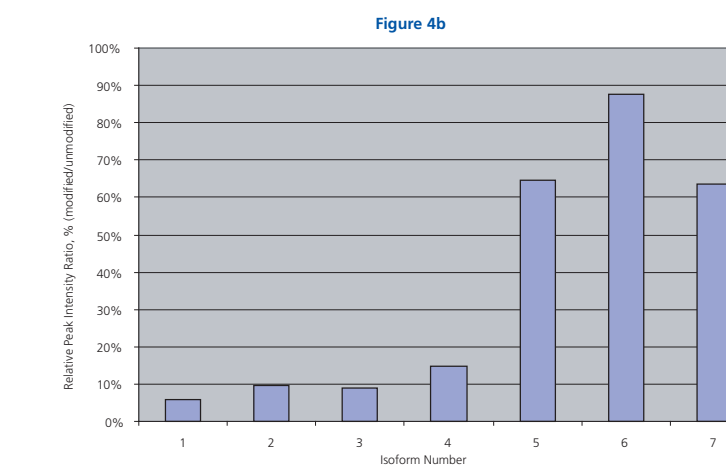
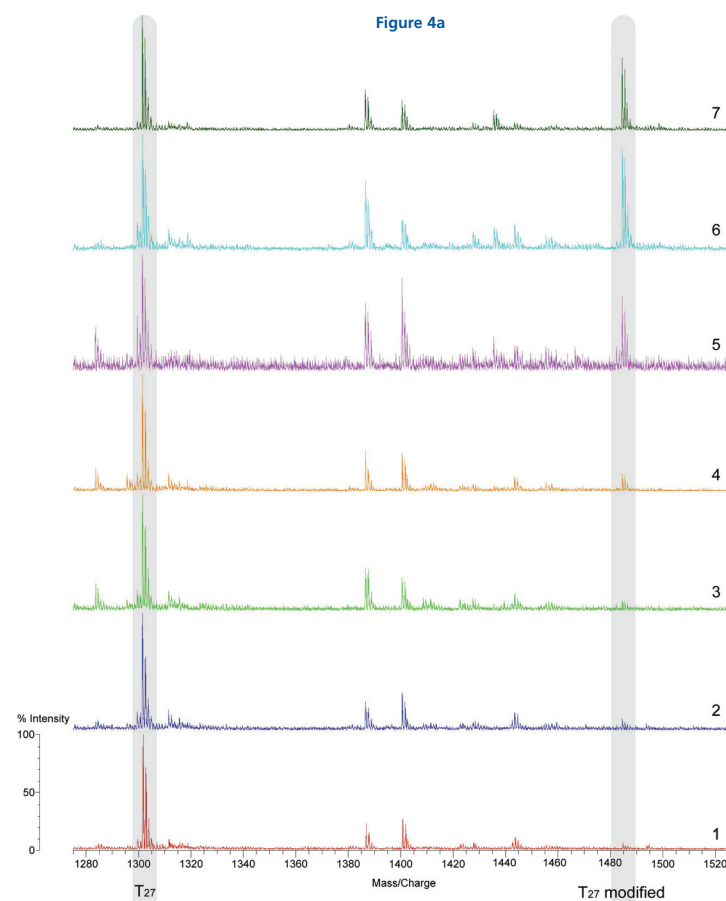


Figure 4: Modification of the Apo A1 1301 MW Peptide by 183 Da

- The relative abundance of one pair of partner peptides (1301 and 1484 Da) was compared (Figure 4a) across the isoform train spot sequence.
- The modified peptide (m/z = 1484) has a markedly lower abundance in isoforms 1-4 relative to elevated levels in isoforms 5-7 (Figure 4b).

Positive ion reflectron MALDI mass spectra are shown in the m/z range of 1275-1525 for Apolipoprotein A1 isoforms 1-7 (low to high pI respectively). All spectra are normalized to 100% of the unmodified T₂₇ peak intensity. The relative peak intensity ratio is calculated by dividing the actual m/z signal for the T₂₇mod peak by the actual m/z signal for the unmodified T₂₇ peak, expressed as a percentage.

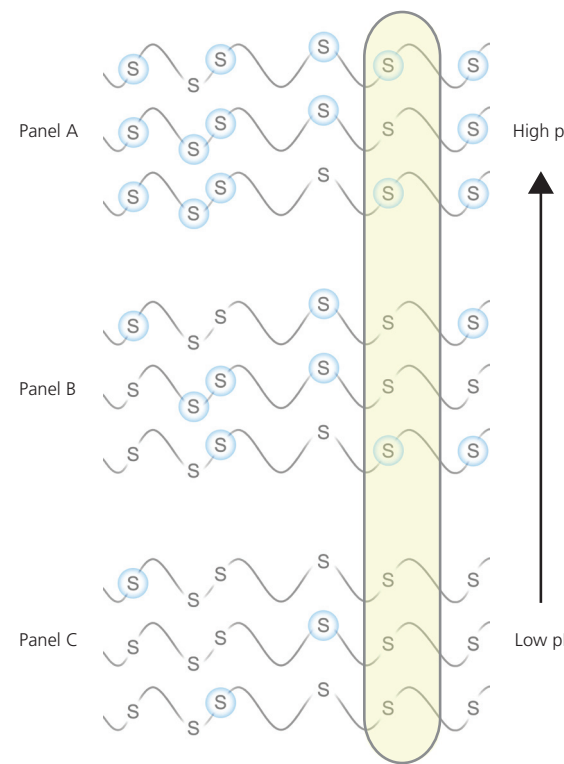


Figure 5: Theory of Peptide Modification Within Isoforms

This illustration represents three sets of the peptide chain of Apo A1. The "S" represent unmodified native serine residues, whereas the circled "S" represent AEBSF-modified residues. The shaded band corresponds to a hypothetical tryptic peptide incorporating a single serine residue. The three-peptide chains in each panel have the same overall number of modified serine residues, and therefore are constituents of the same isoform. The total number of modified (circled) serine residues decreases from Panel A to Panel C, indicating the variation between the different isoforms present in the train. As illustrated, Panel A represents the highest pI isoform, and Panel C represents the lowest pI isoform. The shaded band highlights the model of a hypothetical tryptic peptide detected in the mass spectra having a mixture of both modified and unmodified peptides. Isoforms differ according to the charge carried by each protein, and as such each isoform must be comprised of proteins with the same net charge. For proteins within each isoform, it is postulated that the net number of charge-inducing modifications is constant, but their location on the protein is randomized. Hence, "occupancy" of a potential modification site (in this case serine residues) is statistically dependent on the number of modifications, and results in the difference in pI that is observed. This explains the appearance of both modified and unmodified peptides in a single mass spectrum of protein derived from the same pI spot.

Conclusions

- Inclusion of AEBSF in a plasma sample increases the number of higher isoforms of Apo-A1 as seen by 2DE and reduces the overall gel resolution.
- This effect is not due to protease inhibition
- The covalent modification of serine residues by AEBSF adds a positively charged amine group, which shifts the proteins to higher pI forms.
- Sequential modifications of the available serine residues appears to shift the protein to the next highest pI spot.
- Certain protease inhibitors such as AEBSF must be used judiciously and with careful thought prior to experimentation if one is to avoid the generation of artifactual isoform spots in protein trains.

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