On Line supplement

A Specific Proteinase 3 Activity Footprint in Alpha-1-antitrypsin Deficiency

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Online Data Supplement

Identifying the PR3 Cleavage Site

Fibrinogen is a physiological substrate for PR3 and initially, specific fibrinogen cleavage products generated by PR3 (Athens Research and Technology, Athens, GA) were identified by mass spectrometry (MS) and gel electrophoresis as described below.

Matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) analysis (UltrafleXtreme, Bruker) detected a peak in a PR3/fibrinogen digest solution (red peak) but not an NE/fibrinogen digest (green peak). This peak was also present in plasma from patients with AATD (blue profile) as shown in figure E1.

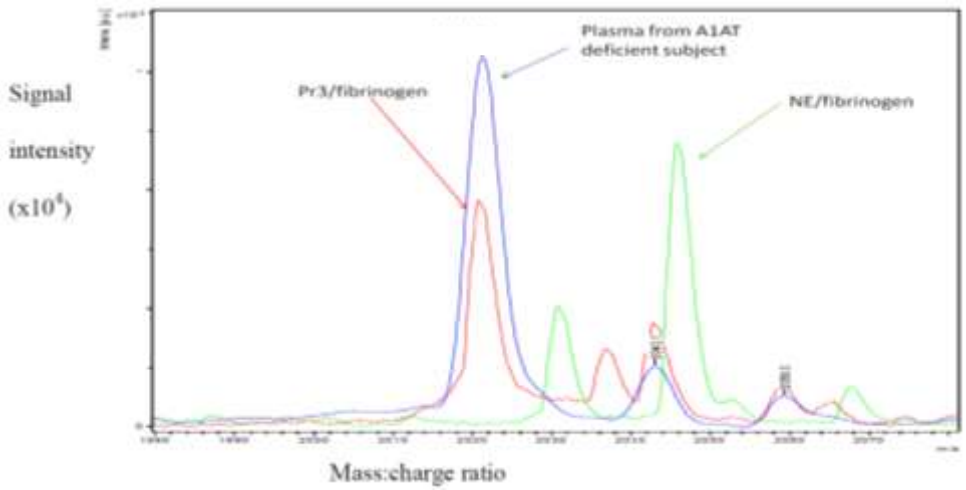

Figure E1. *An example of MS Analysis of a Proteinase 3/fibrinogen digest solution shown in red detected a peak which was also present in plasma from patients with alpha-1-antitrypsin deficiency shown in blue but was not present in a neutrophil elastase/fibrinogen digest solution shown in green.*

Reducing gel electrophoresis demonstrated several bands in both cleaved and uncleaved fibrinogen. Some bands (A and B) were present in a PR3/fibrinogen solution but not NE/fibrinogen or uncleaved fibrinogen (figure E2). Size markers are shown and the derived molecular weight of bands D and C are added.

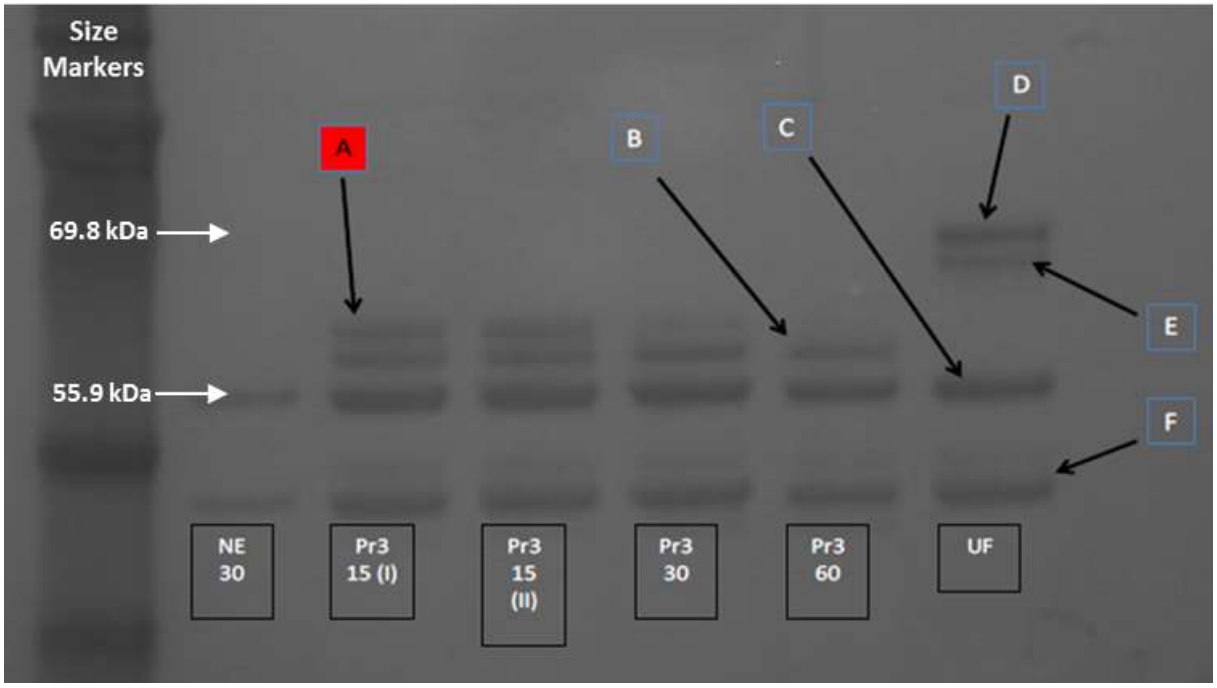

Figure E2. *Polyacrylamide gel electrophoresis of cleaved and uncleaved fibrinogen (UF). Neutrophil elastase (NE) or Proteinase 3 (PR3) was incubated with fibrinogen for various lengths of time (15, 30 or 60 minutes). Labels I and II are from two separate incubations, both for 15 minutes. Bands of individual sizes are labelled A-F. Bands D and E both represent isoform 2 of the complete fibrinogen alpha chain. NE and PR3 alone would migrate with a mass of 29kDa.*

Subsequent digestion of band A showed that it was generated by cleavage of the fibrinogen alpha chain on the carboxyl side of K527 by PR3 (see figure E3). Further gel electrophoresis and MS analysis demonstrated that the molecular weight of band A is between 56-70kDa suggesting the C-terminus lies between M536 and G548 (underlined). The trypsin (yellow) and Asp-N (blue) cleavage sites closest to the likely C-terminus (bold) also suggested that the PR3 cleavage site was unlikely to lie beyond R547.

These data support the PR3 cleavage lying between K527 and G548 (trypsin specific cleavage sites). Asp-N digest of band A and subsequent MS analysis detected a number of peaks with varying molecular weights. One peak had a molecular weight 2236.08Da, which is consistent with a peptide fragment between D521 and V541.

These data confirm that valine 541 of the fibrinogen alpha chain (Aα-Val^{541}) is the C-terminus of band A and the site of PR3 cleavage.

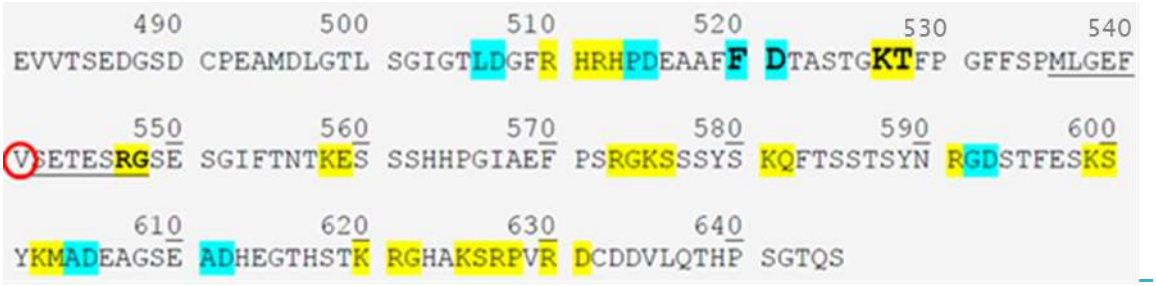

Figure E3. *Peptide sequence of part of the fibrinogen alpha-chain. Sites of trypsin (yellow) and Asp-N (blue) cleavage near the likely carboxyl terminus of band A are highlighted. Those identified by peptide mass fingerprinting are shown in bold. The molecular weight of band A is between* 56-70kDa suggesting its carboxyl terminus lies in the underlined region between M536 and G548.

The size of the fibrinogen cleavage product generated (2236Da; D521-V541) was assumed to have a reasonable circulating half-life and be enzyme specific as no such fragment was produced by NE. The carboxyl terminal six amino peptide sequence to Val^{541} was then selected as the target to develop a specific assay for this cleavage site.

The Aα-Val^{541} Assay

An indirect ELISA was then developed using fibrinogen incubated for 15 minutes at 37°C with PR3 (molar ratio of 1:200 PR3:Fibrinogen and reaction terminated with an excess of 0.1% trifluoroacetic acid) as the antigen to coat 96-well black high bind microplates (Corning, Wiesbaden, Germany) at 4°C overnight. The following day a solution containing 1% bovine serum albumin (BSA) was applied for 1 hour at 37°C to block remaining sites and prevent non-specific binding. Standard peptide (COMLGEFV) from 0-125nM or samples (15µl plasma added to 60µl block buffer) were incubated with the diluted rabbit antiserum (75µl) overnight at 4°C before being transferred to the coated plate washed with tris-buffered saline, 0.05% Tween 20 (TBST). Following 2 hours incubation the plate was washed again with TBST and DELFIA europium-conjugated anti-rabbit IgG added (concentration 806ng/ml; Perkin Elmer, Seer Green, UK). After 1 hour and a further wash DELFIA enhancement solution (Perkin Elmer, Seer Green, UK) was added to develop the fluorescent signal. Fluorescence was read at 340nm excitation and 620nm emission using a BioTek Synergy 2 plate reader running Gen5 software. The difference in signal between application of pure antiserum (B0) and that incubated with varying concentrations of peptide (B) was used to derive a standard curve (3-order polynomial trend line) in Excel from which sample results were obtained by interpolation.

Titration experiments were conducted to establish the optimal concentrations for both PR3 cleaved fibrinogen and rabbit antiserum used in the assay. Final dilutions were 1:20,000 for the antiserum (sufficient stock to measure $3.5x10^9$ samples at this concentration) and 1:16,000 for PR3 cleaved fibrinogen (equivalent to adding ~3.8nM per well). These conditions gave a B/B0 of approximately 16% when using 125nM of peptide, which is comparable to the $A\alpha Val^{360}$ assay described previously [10].

Samples were run in triplicate on each plate and average result taken as for the $A\alpha\text{-}Val^{360}$ assay. Pooled plasma from PiZZ patients was used as a control sample and added to every plate. Sample results were only accepted if the control result was within 2 S.D. of the mean obtained from previous replicate samples. Samples from healthy controls and PiZZ AATD patients were able to be measured using the same standard curve.

Validation of the rabbit antiserum used in the assay is described in further detail in the experiments below.

Rabbit Anti-serum

Antibody (serum) from two rabbits (R3839 and R3840) generated by the bovine thyroglobulin linked peptide described in the main text was tested at various concentrations using the assay design described above. Rabbit R3839 gave a better signal in terms of relative fluorescent units (RFU) especially at higher dilutions indicating higher affinity/concentration. The initial results are shown in figure E4.

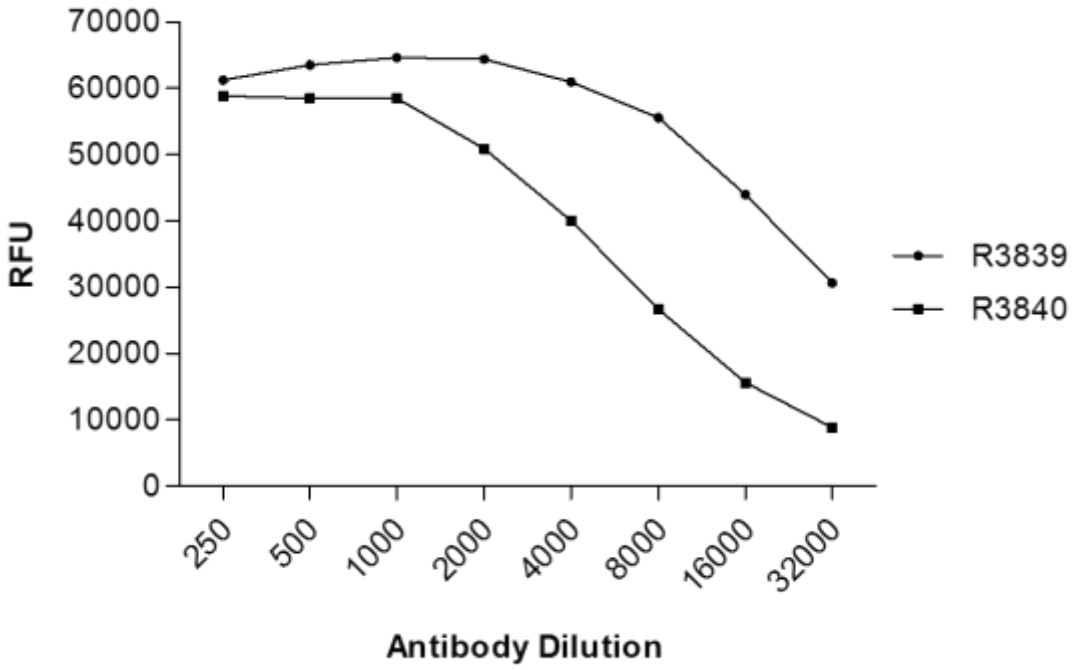

Figure E4. *Comparison of anti-serum from two immunised rabbits (R3839 and R3840). Diluted antiserum (1:250 – 1:32,000) added to a PR3 cleaved fibrinogen coated plate gave higher relative fluorescent unit (RFU) readings for rabbit R3839 and was used for subsequent boosting and antibody harvesting.*

Specificity

Specificity of the antiserum was tested with carboxyl terminal modifications of the peptide used to generate the antibody. The free C-terminal valine was necessary for recognition by the antibody (figure E5).

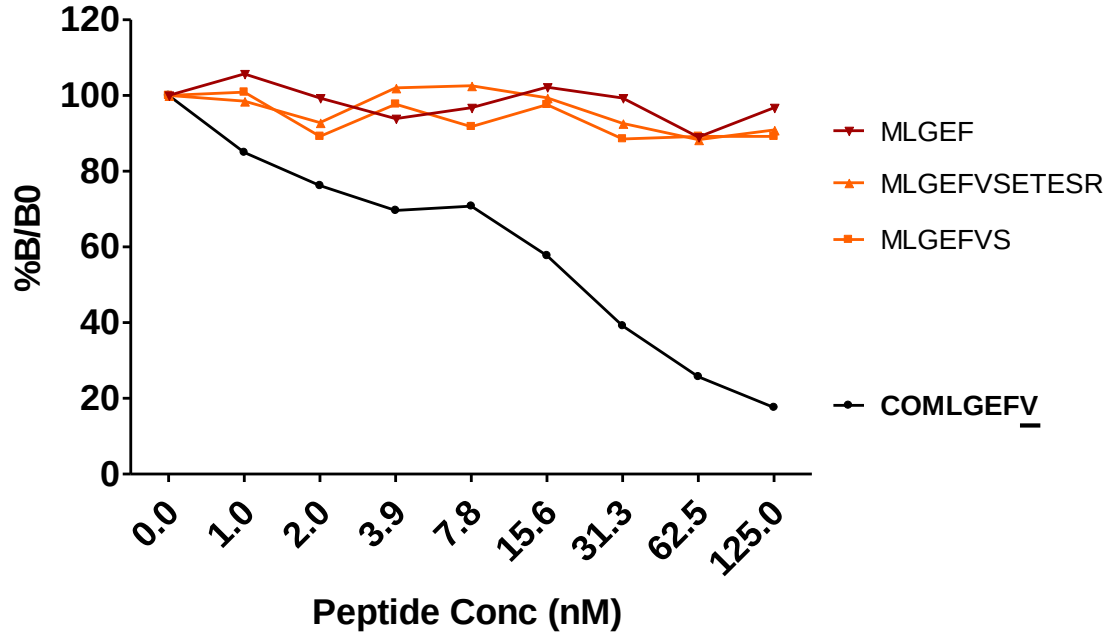

Figure E5. *Specificity of the R3839 antiserum demonstrated by carboxyl terminal extensions (1mer and 6mer) to the COMLGEFV peptide. Loss of inhibition was also seen with the single terminal valine deleted.*

Using Aα-Val^{360} Peptide for the Standard Curve

Aα-Val^{360} is a previously described marker of pre-inhibition NE activity in vivo. The peptide used as a standard in the Aα-Val^{360} assay is CJTSESSV. We also confirmed that this peptide (which also has a terminal valine) was not recognised by the R3839 antibody (figure E6).

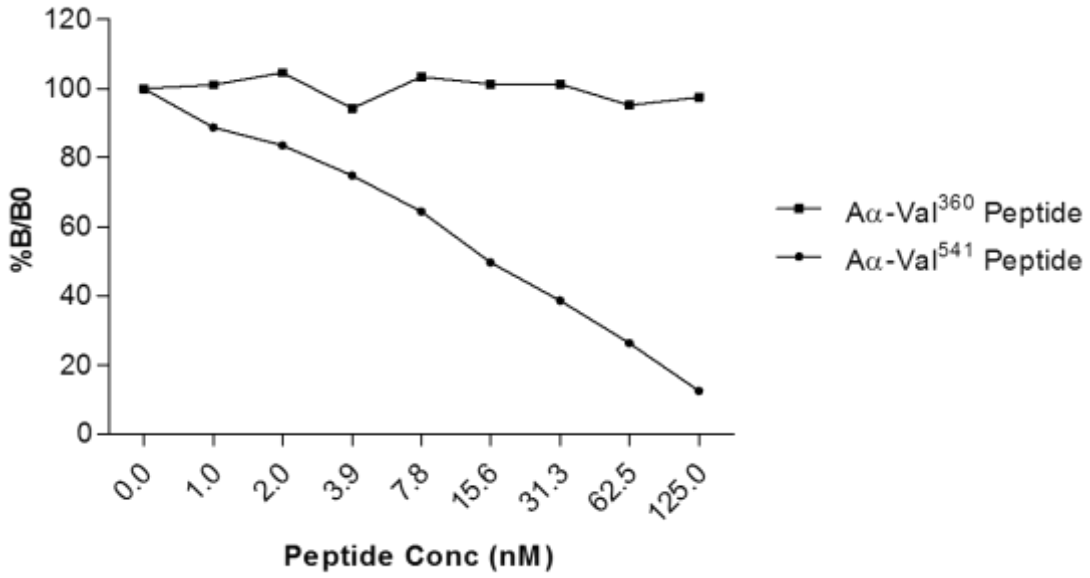

Figure E6. *Incubating peptide CJTSESSV (used as a standard in the Aα-Val^{360} assay) with antiserum from rabbit R3839 does not affect the assay providing further evidence that the antiserum is specific for the Aα-Val^{541} peptide COMLGEFV.*

Measuring PR3 cleaved, NE cleaved or uncleaved fibrinogen

Various concentrations of PR3 cleaved, NE cleaved or uncleaved fibrinogen were added to block buffer and the assay performed as usual.

Virtually all of the antibody was removed by the PR3 cleaved fibrinogen as shown by a low %B/B0 in figure E7. NE cleaved fibrinogen and, to a lesser extent, uncleaved fibrinogen do bind the antibody because the cleavage site is already exposed physiologically in these preparations. However, at the lowest concentrations (7.8 – 15.6nM) antibody binding is minimal in uncleaved and approximately 9 times less for NE cleaved compared to the complete binding of PR3 cleaved fibrinogen.

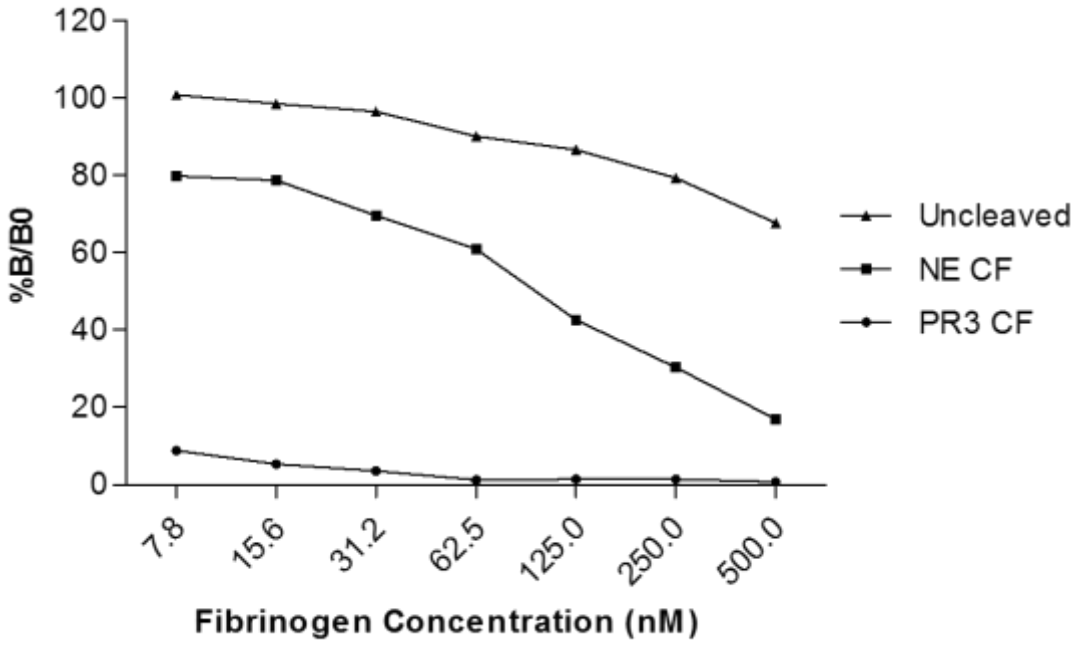

Figure E7. *Uncleaved fibrinogen, neutrophil elastase cleaved fibrinogen (NE CF) and proteinase 3 cleaved fibrinogen (PR3 CF) incubated with R3839 rabbit antiserum and subsequently transferred to a PR3 cleaved fibrinogen coated plate. PR3 CF inhibits all of the antibody present. Uncleaved fibrinogen and NE CF bind the antibody but this is minimal at the coating concentration (7.8nM).*