

Characterization of ARALAST Compared to other A1PI Preparations

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Baxter

Agenda

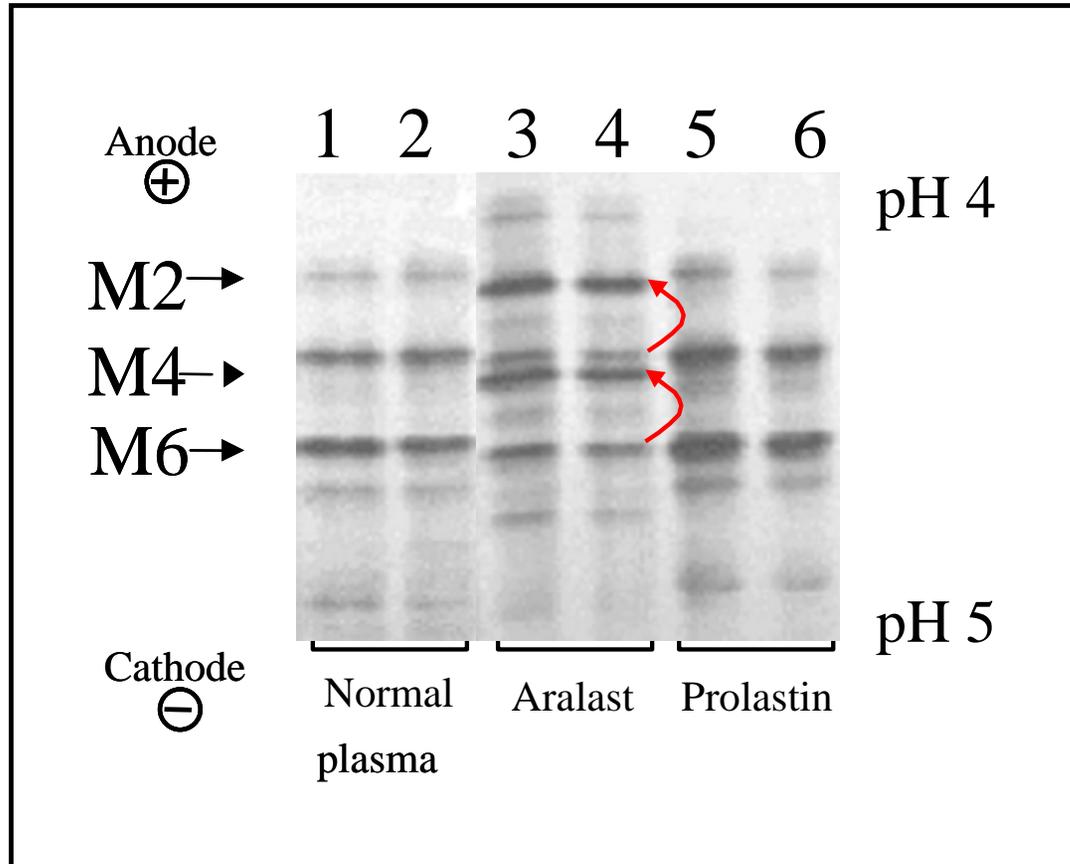
- 1) Background Information
- 2) Modifications to primary structure of A1PI in all commercial products
- 3) Potential cause for microheterogeneity of A1PI protein in Aralast
- 4) Implications of microheterogeneity on protein structure and function

Aralast - Introduction

- ▶ **December 23, 2002:**
FDA approval of Aralast, developed and manufactured by Alpha Therapeutics (now: Grifols Biological Inc.)

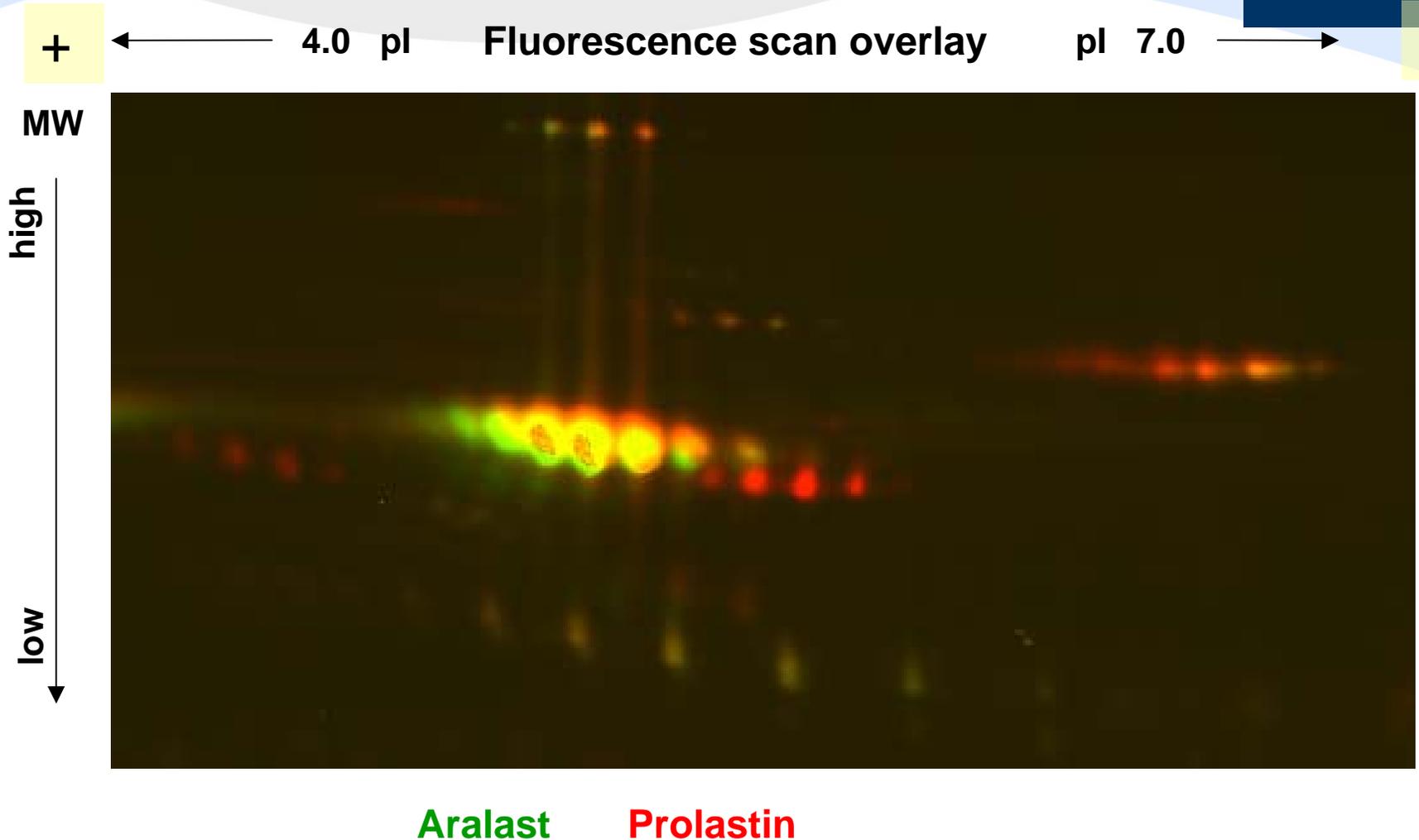
- ▶ **Q2 2003:**
Baxter acquired Aralast and Aralast associated assets from Alpha Therapeutics

IEF Observations:



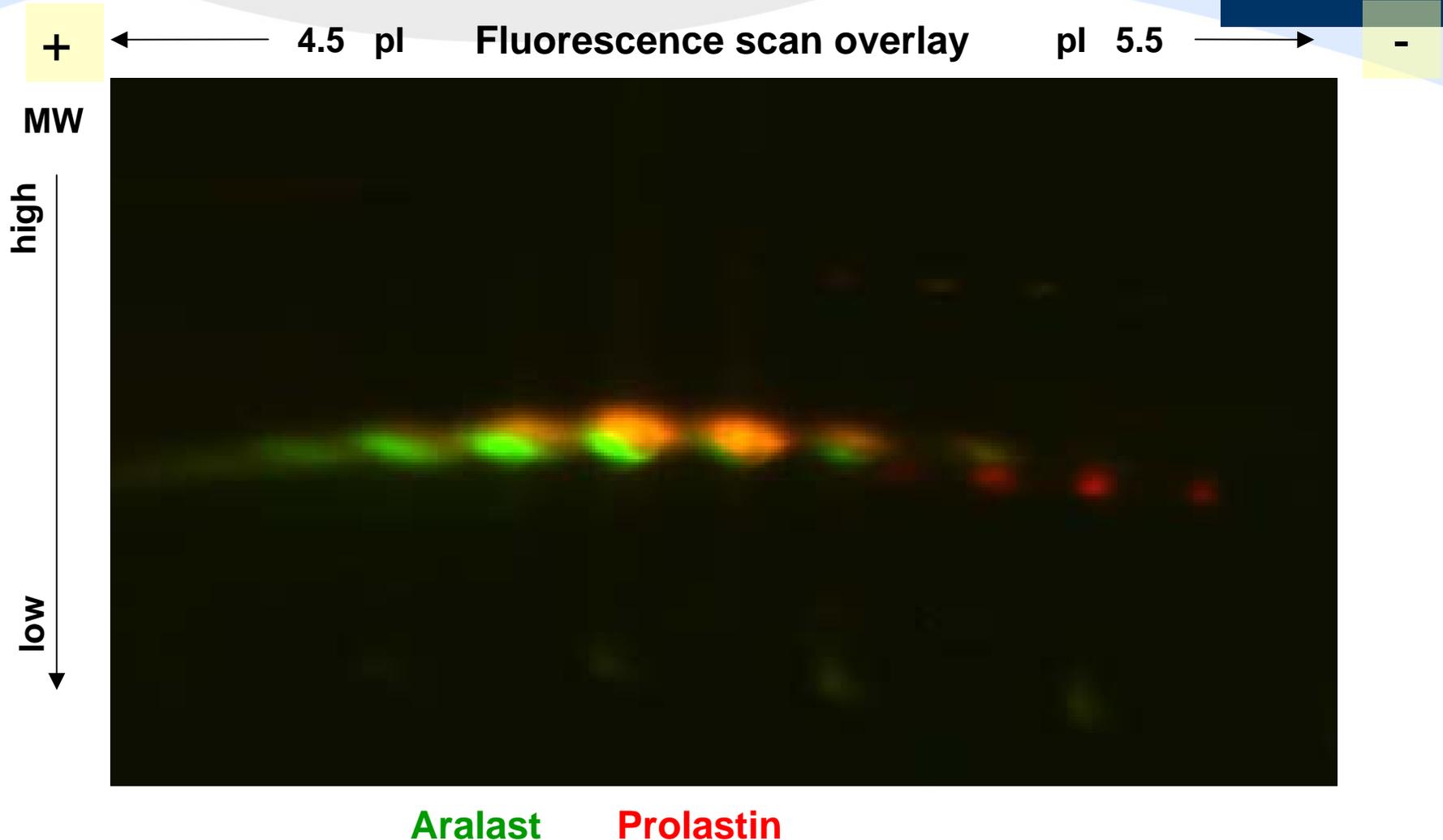
IEF-gel provided by the FDA regarding their concerns of an anodal shift of M6 and M4 in ARALAST, suggesting that a population of AAT isoforms carry approximately one extra negative charge

2DIGE Analysis of Aralast and Prolastin



Anodal shift of spots derived from Aralast seen on 2DIGE analysis in the presence of urea

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Modifications with Potential Impact on Protein Charge

Secondary structure:

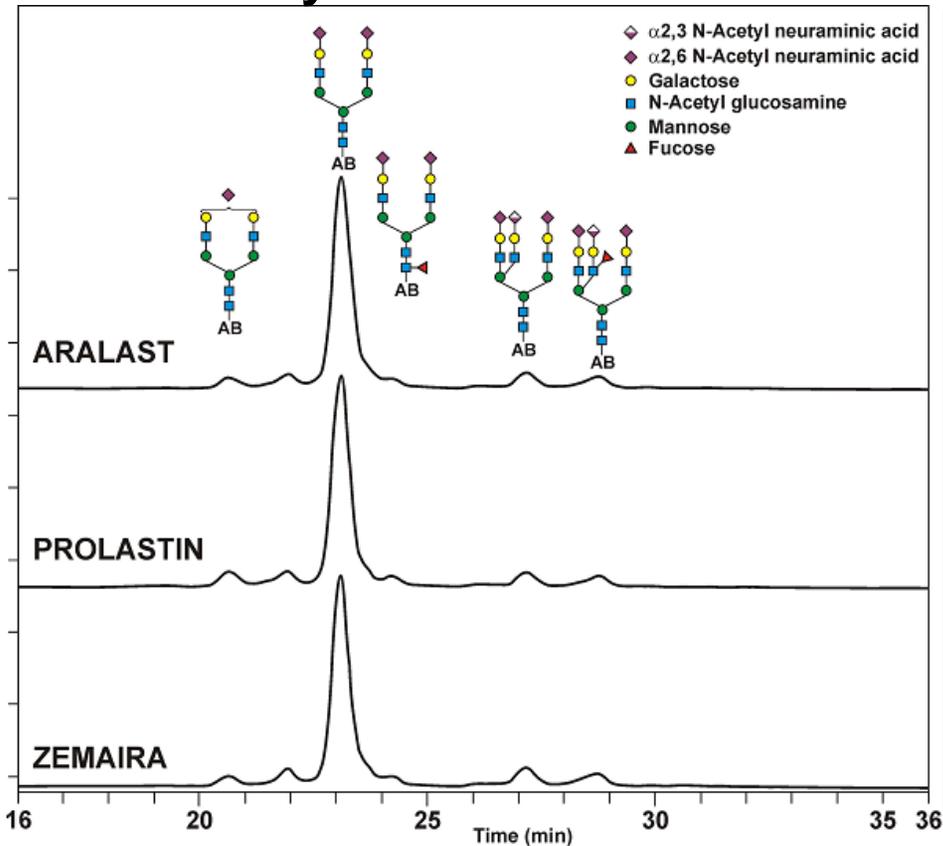
- **Heterogeneity of glycosylation**
 - Influence on the number of sialic acids

Primary structure:

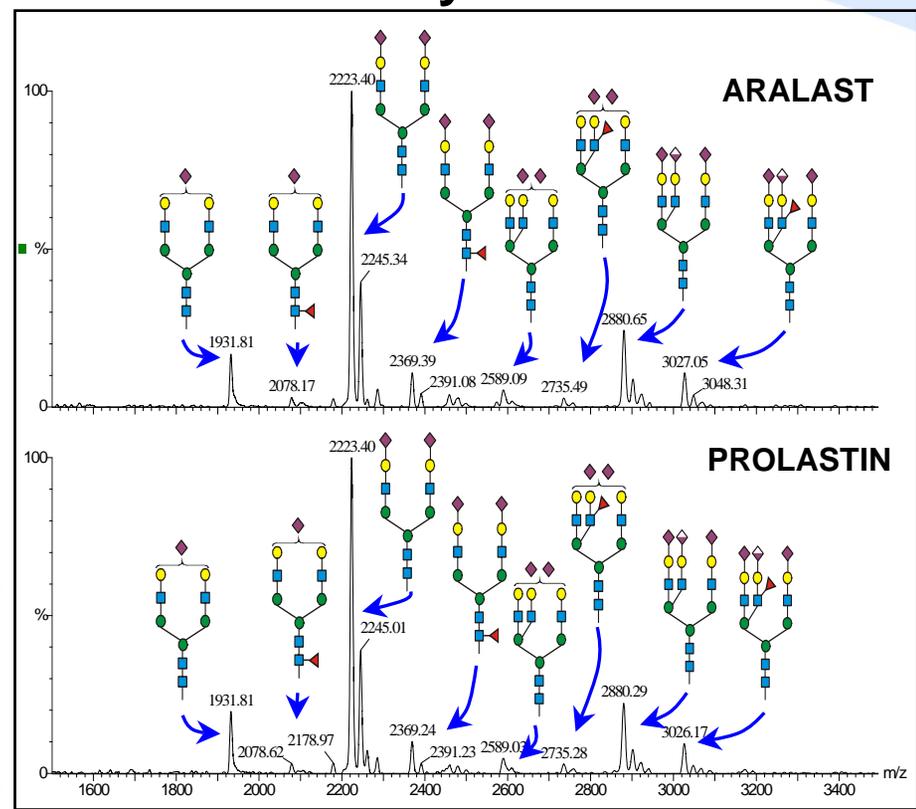
- **Deamidation (addition of a negative charge):**
 - Non-enzymatic conversion of Asn116 or Asn314 to aspartic or isoaspartic acid
- **Cysteine modification:**
 - Attachment (covalent) of another molecule to A1PI (a free available cysteine exists in A1PI)
- **Removal of terminally located charged amino acids**
 - N-terminal truncation of 5 amino acids, loss of **negatively** charged glutamic and aspartic acids → M7, M8
 - C-terminal truncation, loss of **positively** charged lysine ...**des-Lys**
A1PI

N-Glycan Analysis of A1PI concentrates

HPLC Analysis



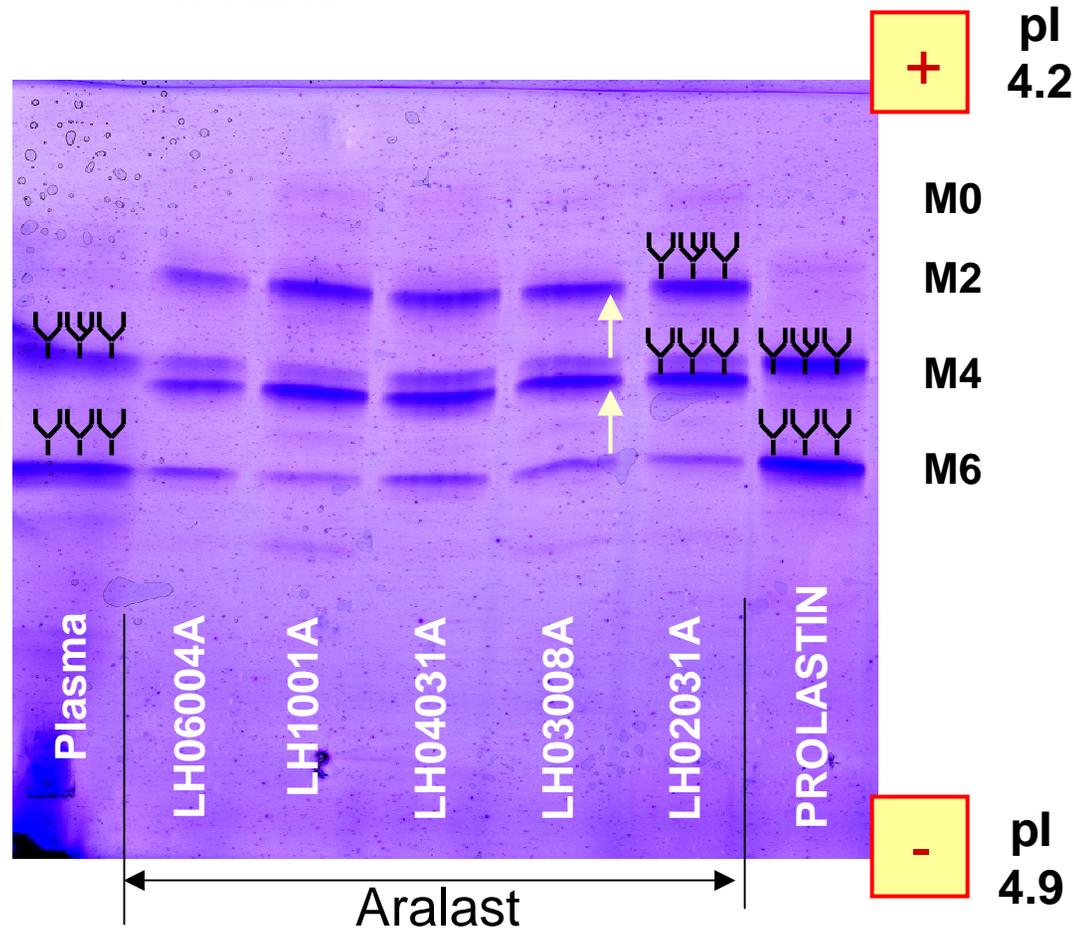
MALDI-TOF Analysis



No difference in the N-glycan profile between Aralast, Prolastin and Zemaira and the pattern is similar to that found for A1PI from human plasma

N-glycan pattern of all 3 A1PI concentrates used for treatment of Hereditary Emphysema is similar to that of plasma, therefore ethanol fractionation and downstream purification have no impact.

Relation of N-glycan pattern to IEF bands



N-glycans are **NOT** responsible for the IEF pattern characteristic for Aralast

Modifications with Potential Impact on Protein Charge

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- Heterogeneity of glycosylation
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- Quantitative measurement of deamidation using an enzymatic assay (IsoQuant Kit, Promega) based on the methylation of **iso-aspartate**, a final product of Asn-deamidation

Product	% of molecules with one iso-aspartate
Aralast (3 lots)	6%
Zemaira (1 lot)	7%
Prolastin (1 lot)	10%

- Confirmed qualitative detection of deamidation in all products by MS-analysis of two deamidation candidate tryptic peptides containing the sequence Asn-Gly (peptides 102-125 and 311-331)

Deamidation as primary sequence modification occurs in all A1PI concentrates

Modifications with Potential Impact on Protein Charge

Secondary structure:

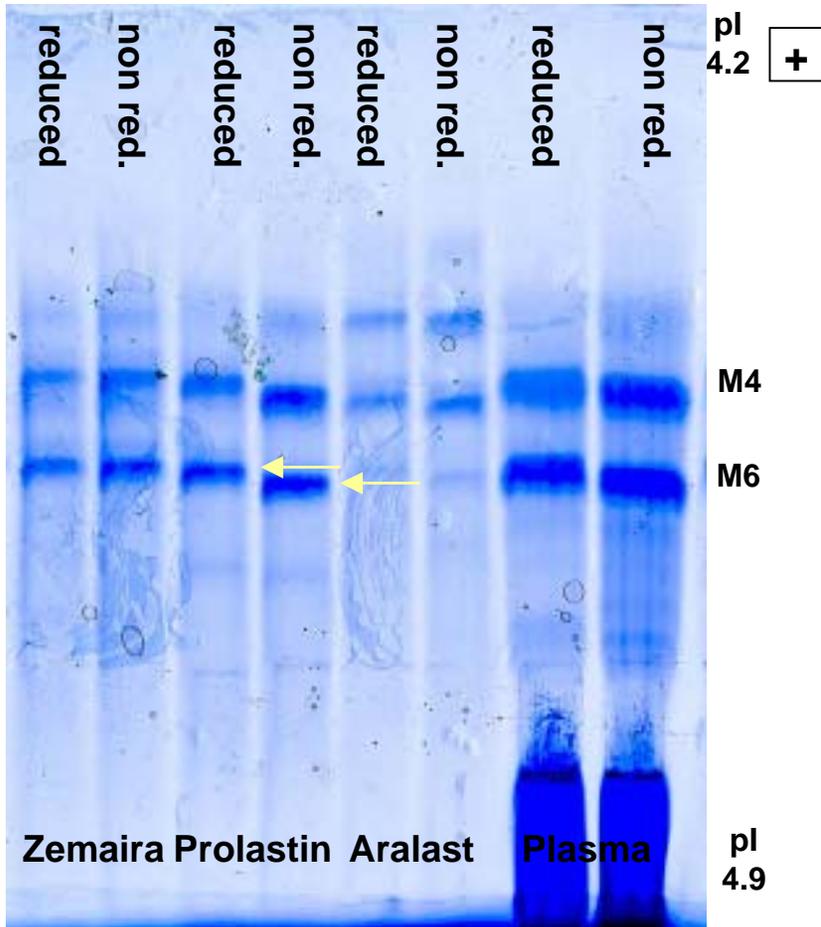
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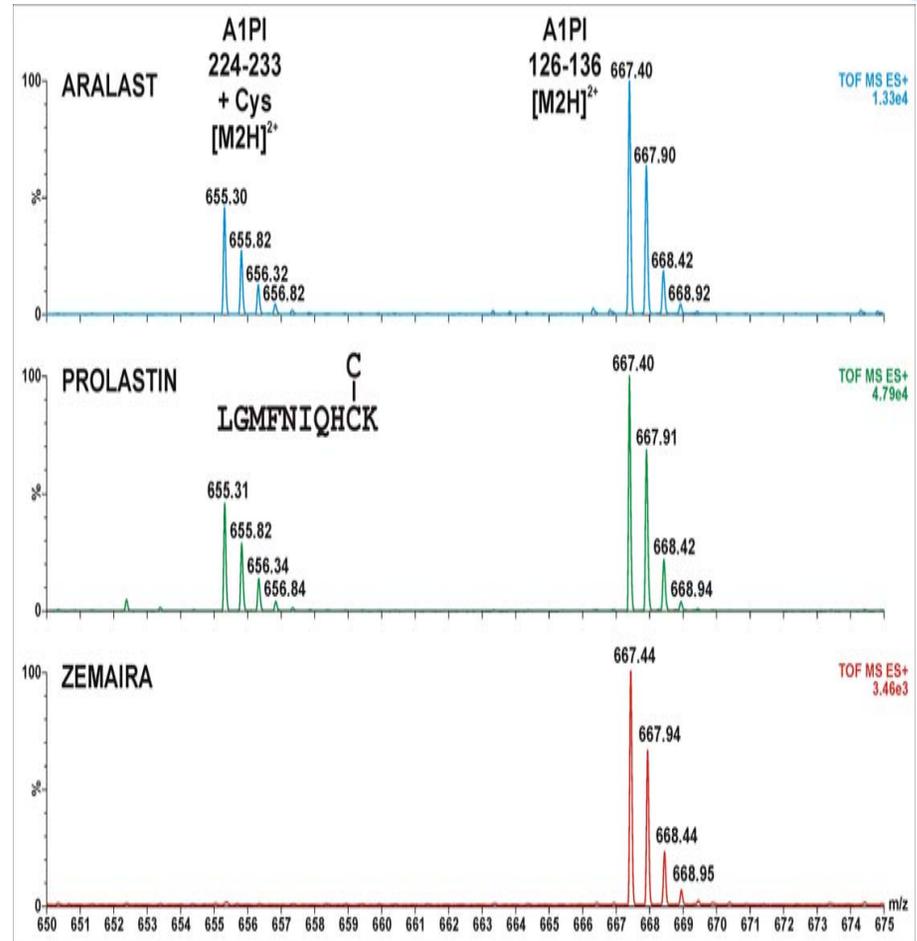
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A1PI

Cysteinylation of Cys232

IEF Analysis



MS-Analysis



Aralast, Prolastin, and A1PI from plasma all exhibit cysteinylation on Cys232, however this modification was not detected in Zemaira

Modifications with Potential Impact on Protein Charge

Secondary structure:

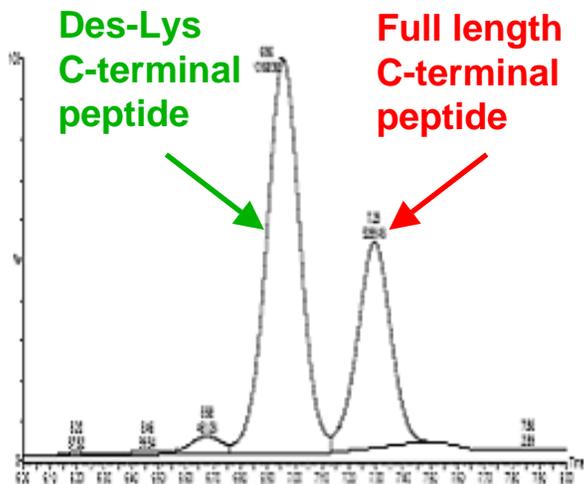
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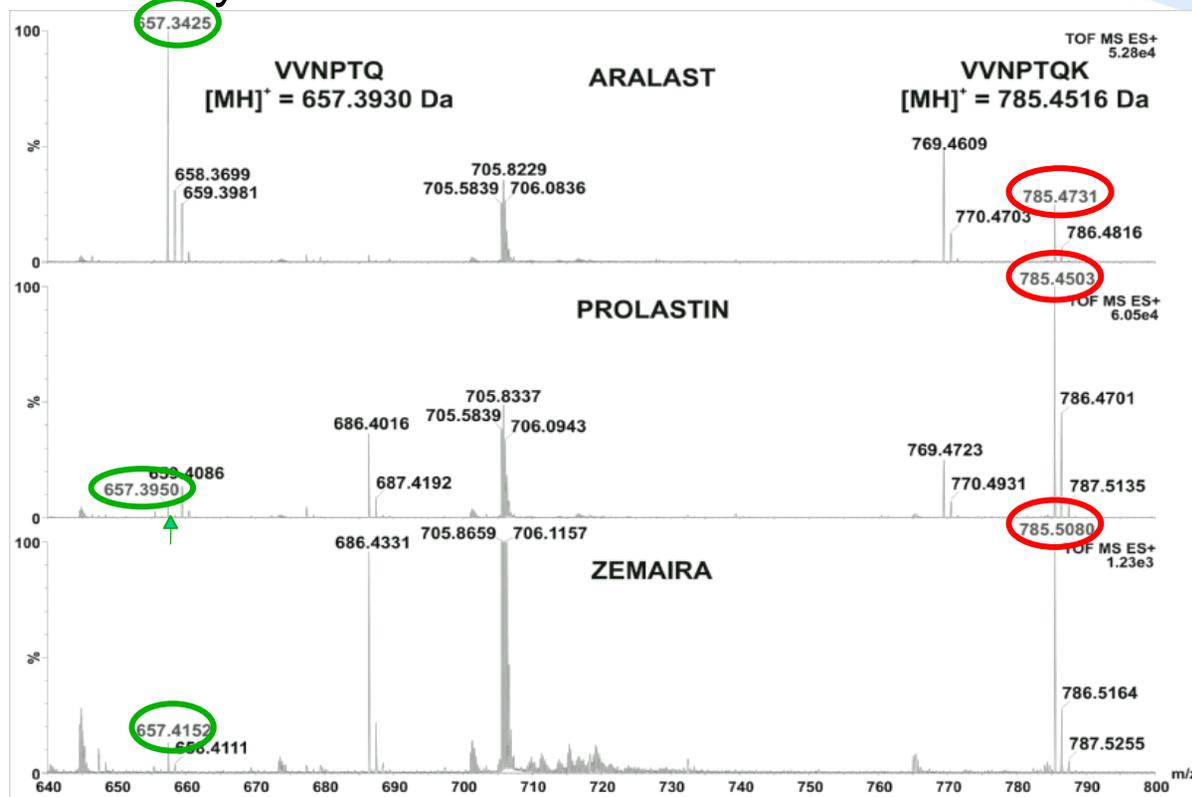
C-terminal Lys Truncation Analysis

HPLC Analysis



Detail of the Aralast tryptic peptide map (detected at 214 nm) showing the region with the C-terminal truncated peptide and allowing quantitation of the modification.

MS Analysis



MS spectra showing C-terminal peptides of A1PI

Des-Lys A1PI found in all products:
Aralast (67%), Zemaira (6%) and Prolastin (2%)

Summary of A1PI Modifications

	<u>Aralast</u>	<u>Prolastin</u>	<u>Zemaira</u>	<u>Plasma/BAL</u>
Glycoisoforms		no major difference		* ₁
Deamidation		detectable		
- qualitative				
- quantitative	6%	10%	7%	not possible
Methionine oxidation	not observed (A1PI fully functionally active)			
C-terminal Lys truncation	67%	2%	6%	? * ₂
Cys232 cysteinylation	+	+	-	+

*₁ Site specific N-Glycan patterns analyzed for the first time; detection of tetra-antennary structures and Lewis X structures on A1PI

*₂ under investigation

→ A1PI in all products differs from A1PI found in plasma

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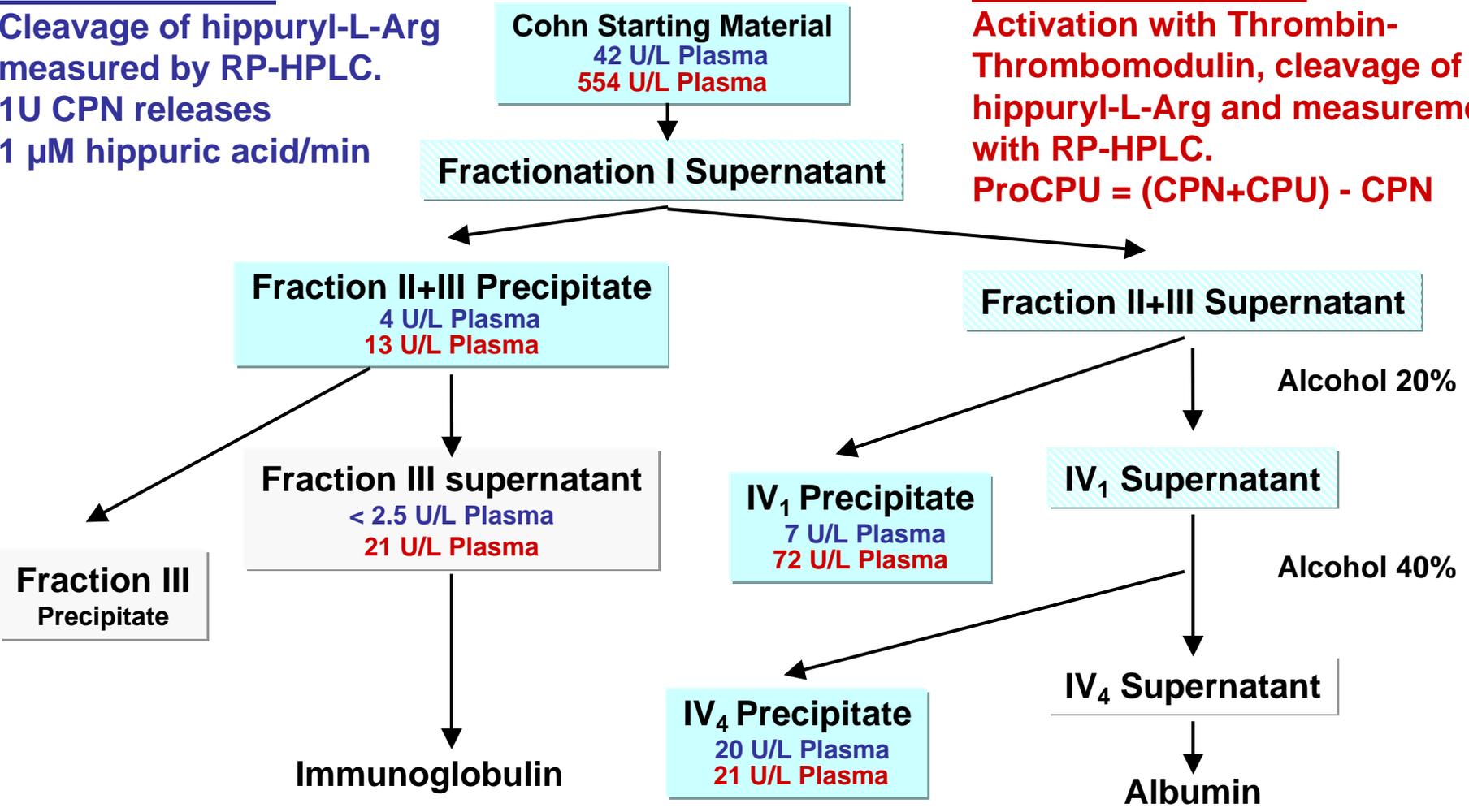
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- **Basic carboxypeptidases are enzymes that cleave COOH-terminal basic amino acids **lysine** and **arginine** from different peptides and proteins**
- **They are involved in food digestion (CPB), modulation of peptide activity (CPM, CPN), pro-hormone processing (CPD, CPE), regulation of the plasminogen system (CPU)**
 - **Carboxypeptidase B (pancreas)**
 - **Carboxypeptidase U**
 - present in plasma as a pro-enzyme, pro-CPU
 - synonyms: CPU = carboxypeptidase R = TAFIa (TAFI = pro-CPU)
 - activated by thrombin, plasmin and trypsin
 - potent inhibitor of fibrinolysis, possibly involved in inactivation of activated complement proteins and anaphylatoxins
 - **Carboxypeptidase N**
 - plasma enzyme constitutively active in plasma, 30 µg/mL plasma
 - inactivation of activated complement proteins C3a, C4a and C5a and bradykinin; maturation of hormones
 - **Carboxypeptidase M**
 - GPI-anchored membrane protein, **highly expressed in lung tissues**

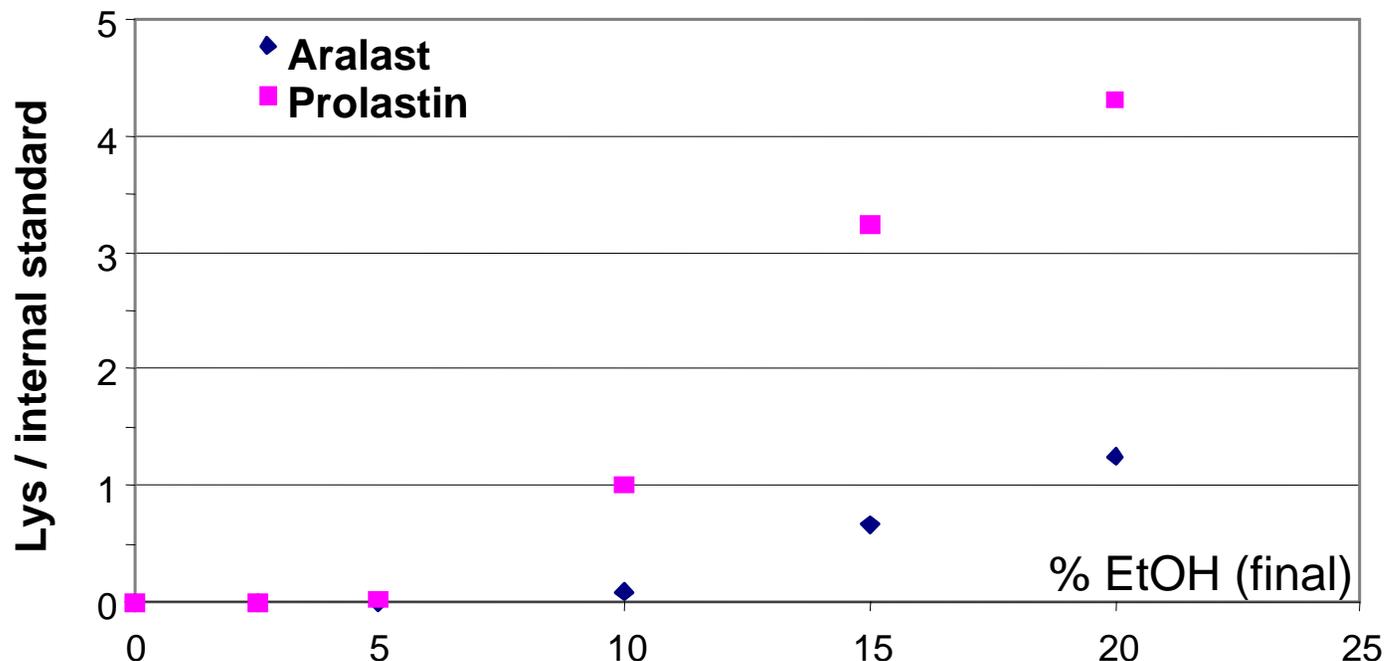
CPN and Pro-CPU Activity in Cohn Ethanol Fractionation

CPN test method:
 Cleavage of hippuryl-L-Arg measured by RP-HPLC.
 1U CPN releases
 1 μM hippuric acid/min

ProCPU test method:
 Activation with Thrombin-Thrombomodulin, cleavage of hippuryl-L-Arg and measurement with RP-HPLC.
ProCPU = (CPN+CPU) - CPN



Ethanol Dependence of C-terminal Lys Cleavage by CPN

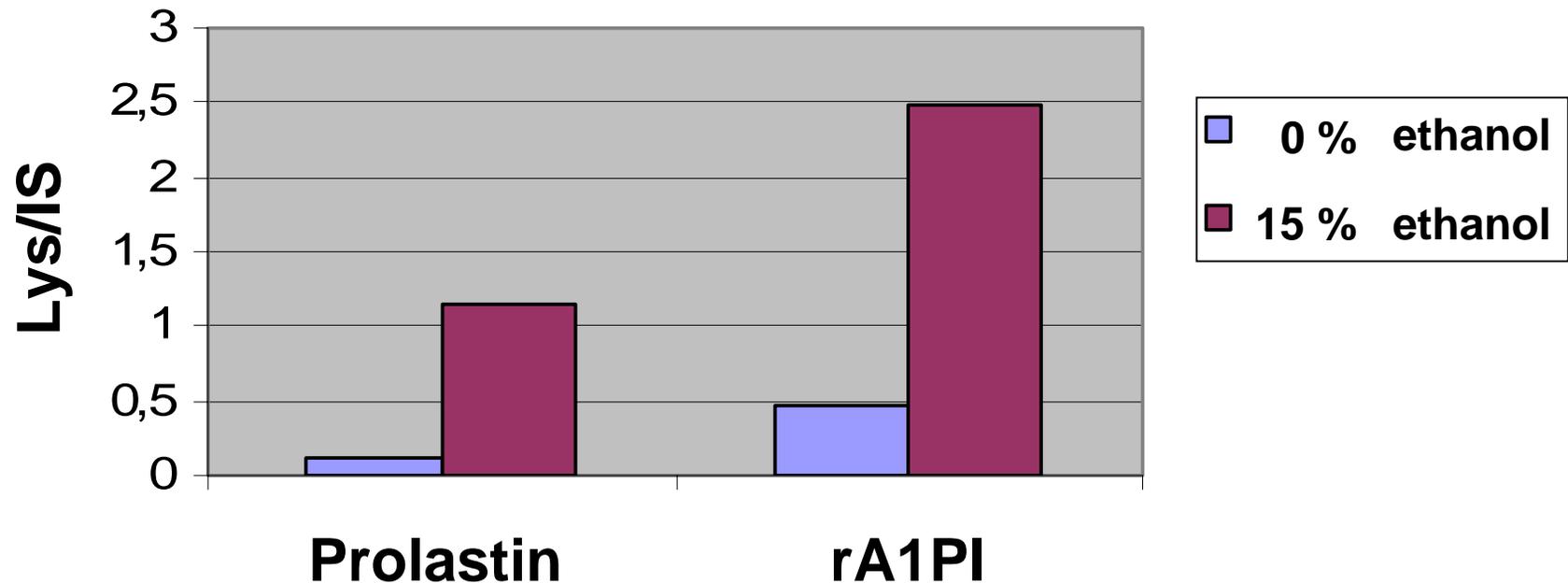


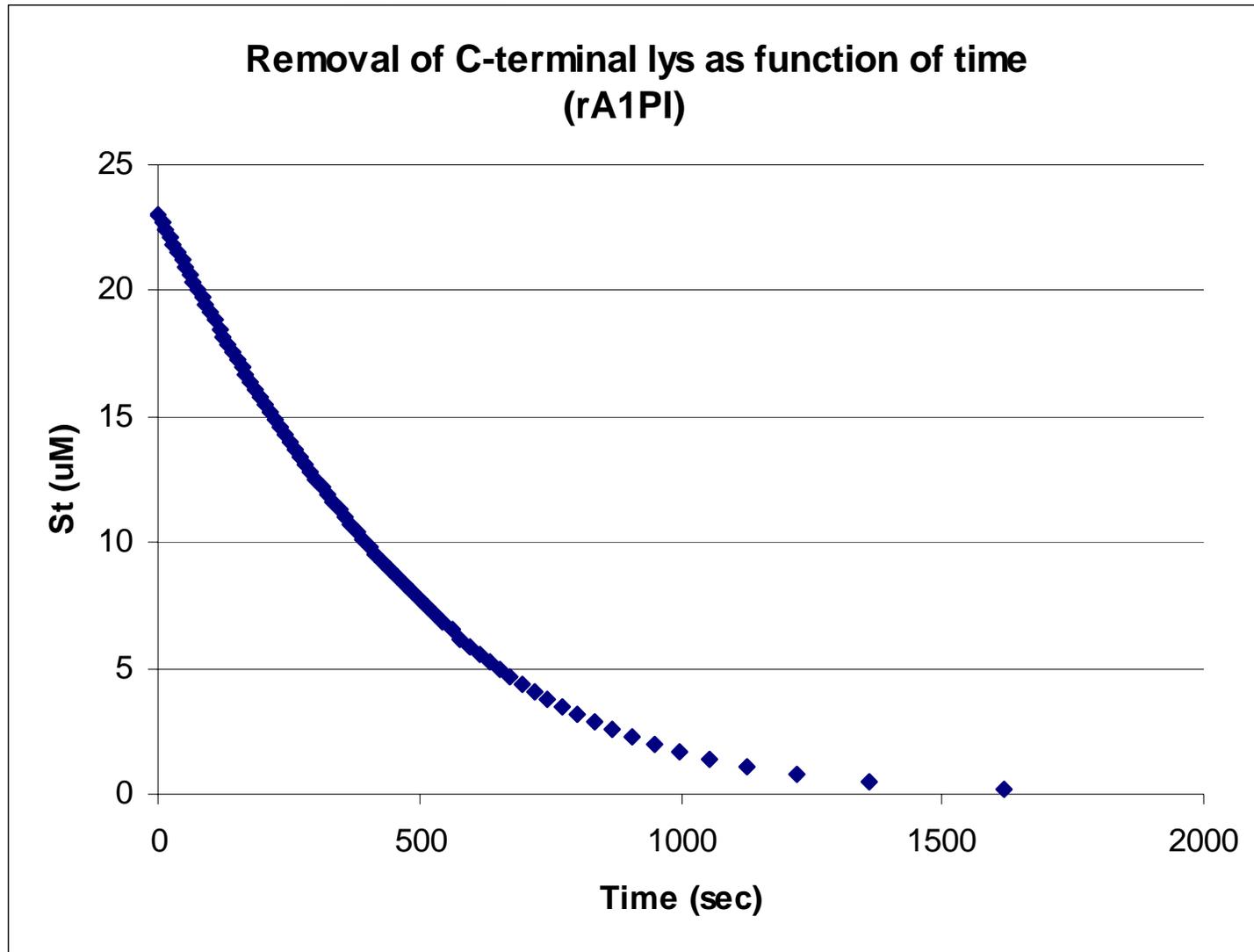
- At EtOH concentrations of $\geq 10\%$ lysine cleavage showed a linear increase in dependence of the EtOH concentration
- The C-terminal Lysine of A1PI in both Aralast and Prolastin are susceptible to cleavage upon exposure to ethanol
- IEF analysis of these samples corroborates lysine truncation to anodal band shift

→ The concentration of EtOH determines the amount of Lys-truncation

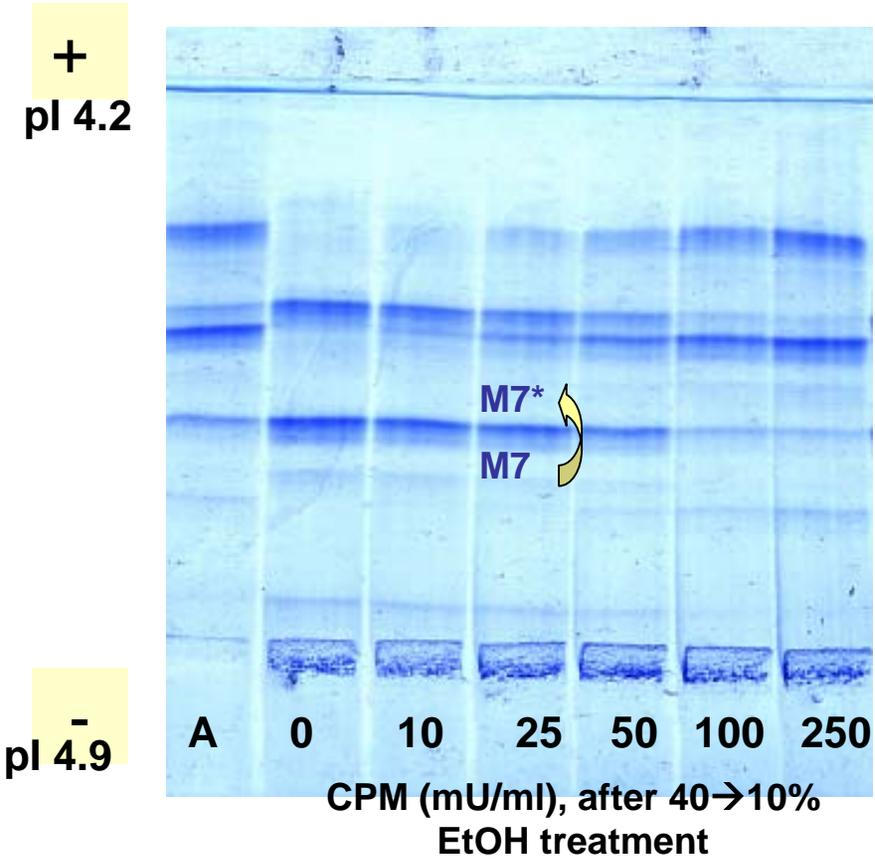
C-terminal lysine cleavage of A1PI by CPN in absence and presence of ethanol

Expressed as ratio: Lysine cleaved/internal standard

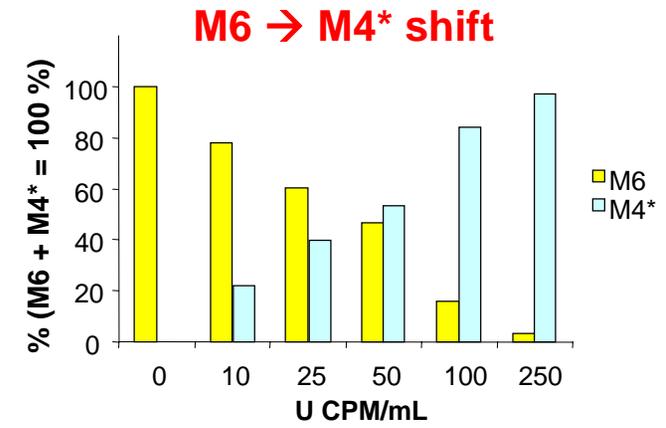
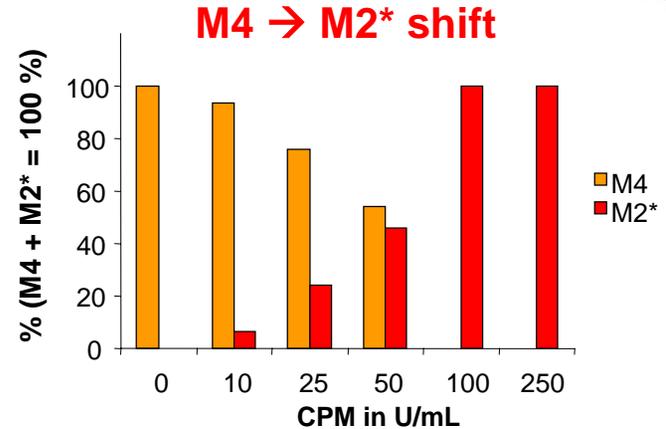




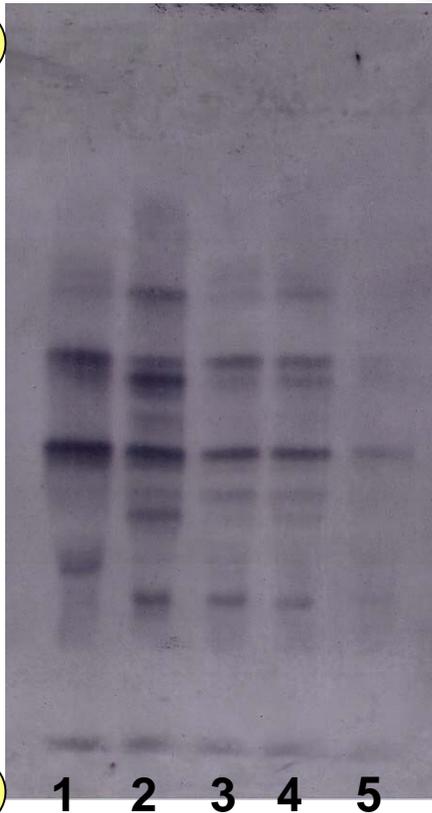
Generation of Anodal Isoforms of A1PI by Treatment with rCPM



Aralast (A) LH02031A
Experimental Lot 900304B (IV,1)



A1PI Isoform Pattern in Human BAL

+**-**

A highly sensitive IEF gel was used to detect A1PI in BAL samples from subjects not on A1PI augmentation therapy

- A1PI was detected in all BAL samples, and the IEF pattern resembles the A1PI shift observed for Aralast
- This suggests that an isoform shift can naturally occur, possibly induced by CPM

1 ... PROLASTIN #PR4HA43A, 5 µg/ml

2 ... ARALAST #LH03002A, 5 µg/ml

3 ... Human BAL sample, 3.5 µg/ml

4 ... Human BAL sample, 6 µg/ml

5 ... Human BAL sample, 2.7 µg/ml

CP Effect on A1PI: Summary

- All basic CPs cleave C-terminal lysine from A1PI
- Cleavage of C-terminal Lys occurs in absence of ethanol
- Ethanol enhances the reaction CPN (20-fold effect of 15% ethanol on k_{cat}/K_m)*
- CPN is the most likely candidate causing the C-terminal Lys cleavage in plasma

* Similar to findings reported by Folk et.al. JBC 1962, vol. 237 pg. 3105 “Kinetics of Carboxypeptidase B Activity - Effects of alcohol.”

Agenda

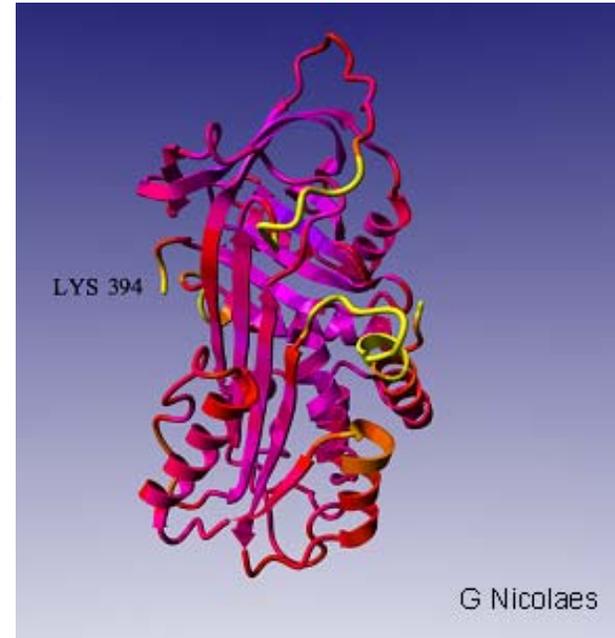
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Implications of Higher Degree of C-terminal Truncation in Aralast: Investigation

- Structural bioinformatics analysis
- In vitro function: anti-elastase activity
- Pharmacokinetics
- Tissue distribution and diffusion

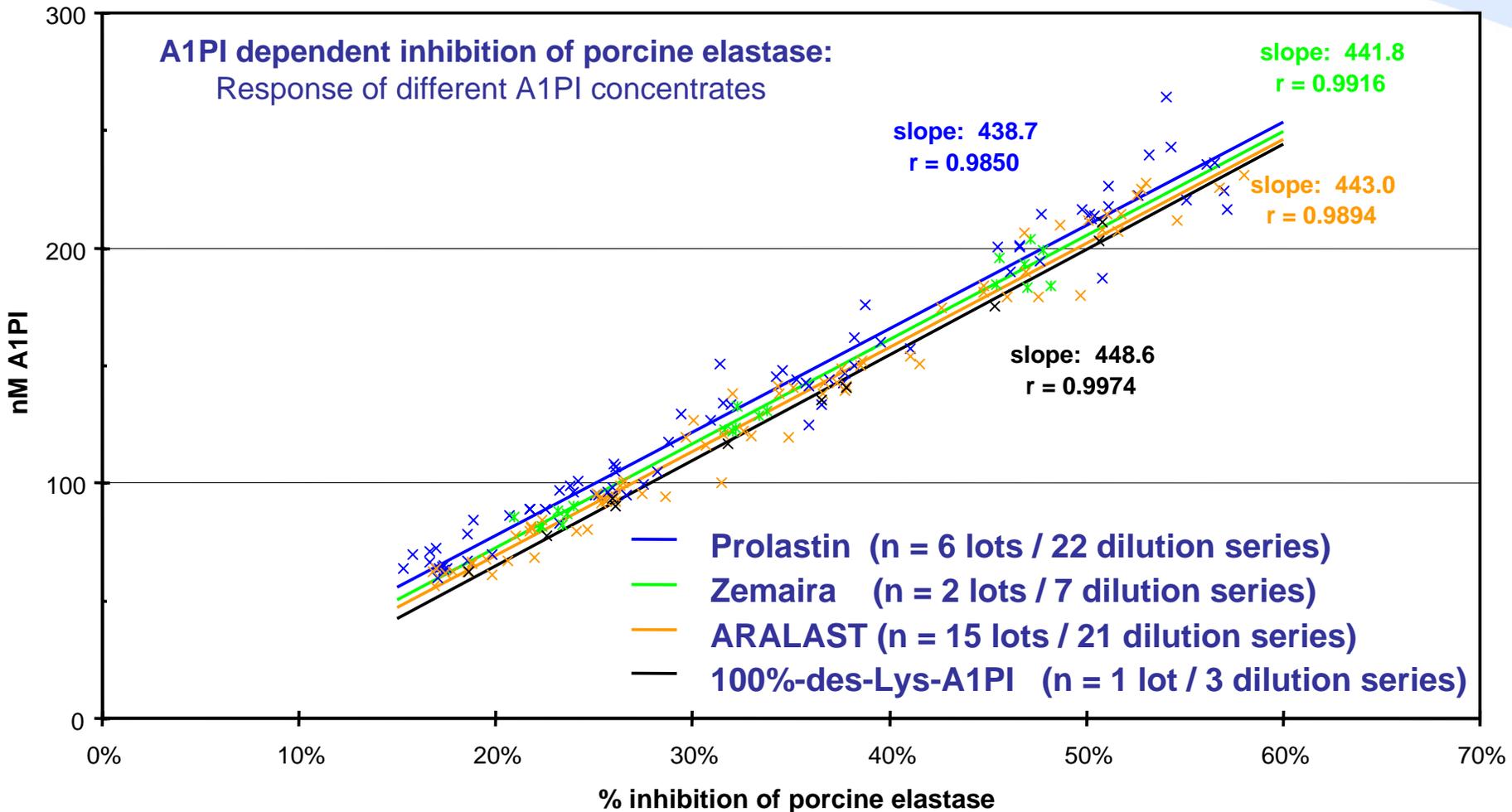
Results

- There is no conservation between species of the C-terminal Lys in A1PI → Lys394 is unlikely to play a major structural or functional role
- 3D analysis of available structures for A1PI and its complex with protease do not support a major structural role for Lys394
- C-terminal loop region is stabilized by an H-bond network in which Lys394 is not involved



→ Lys394 plays no major structural role in A1PI when uncleaved and cleaved as well as when forming complexes with a protease

Functional Relevance of C-terminal Truncation of A1PI



➔ **No difference in activity of Aralast, Prolastin, Zemaira & des-Lys A1PI**

Higher Degree of C-terminal Truncation has no Impact on:

➤ **Metabolic clearance in rats**

→ Comparability of A1PI-preparations with differing degrees of C-terminal lys-truncation

➤ **Distribution to lung (rat)**

→ Comparability of A1PI-preparations with differing degrees of C-terminal lys-truncation including a 100% Δ -Lys A1PI in a rat BAL study

➤ **Diffusion from vasculature into interstitium (guinea pig)**

→ Comparability of A1PI-preparations with differing degrees of C-terminal lys-truncation including a 100% Δ -Lys A1PI in a guinea pig suction blister model

➤ **Confocal studies to assess diffusion and lung tissue distribution**

→ Comparability of A1PI-preparations with differing degree of C-

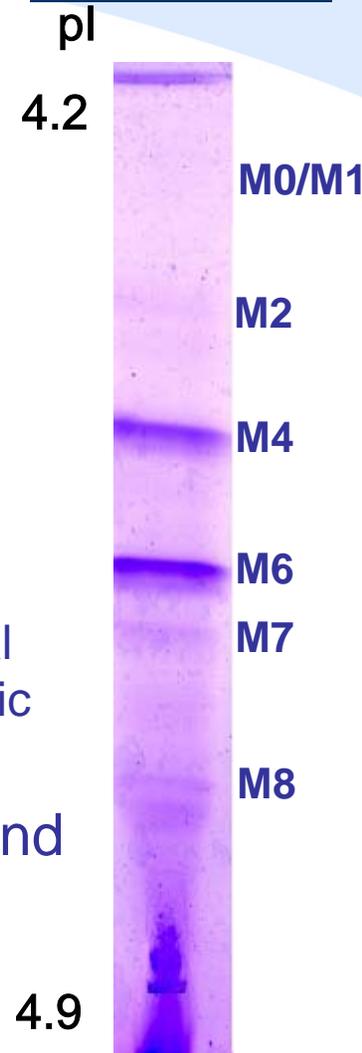
Conclusions

- 1) A1PI in all products approved for augmentation therapy demonstrate at least one primary structure modification (deamidation, cysteine modification, and C-terminal lysine truncation)
- 2) The des-Lys A1PI is induced by carboxypeptidases, and the ubiquitous presence of carboxypeptidases in plasma and in lung tissue (CPM) will likely result in exposure and hence tolerance to the des-Lys394 form of A1PI
- 3) des-Lys is one of many known isoforms of A1PI that does not affect the inhibitor activity, immunogenicity or essential functions of A1PI

REFERENCE SLIDES

Molecular characteristics of A1PI

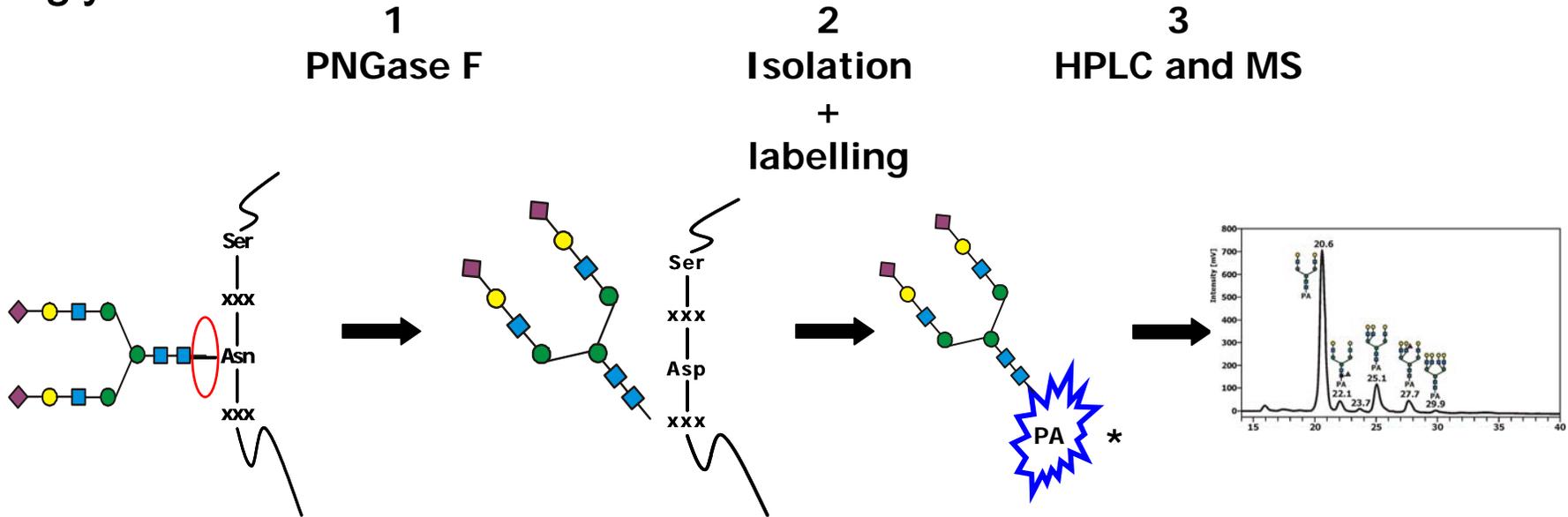
- Single chain glycoprotein consisting of 394 AA
- Carries a high negative charge because of sialic acid residues on three complex glycans attached to Asn46, Asn83 and Asn247
- Exhibits multiple bands reflecting microheterogeneity upon isoelectric focusing (M1 [M0] anodal-low pI to M8 cathodal–high pI)
 - Two minor cathodal isoforms, M7 and M8, are truncated at the N-terminus lacking five AA (1-5) leading to an additional cathodal shift due to the **loss of negatively charged** glutamic and aspartic acid
- 1 single Cysteine residue in position 232 covalently bound to either free Cys or Glutathione via a disulfide bridge
- Asn116 and Asn314 are susceptible to deamidation (Asn → Asp) due to sequence as followed by Gly



HPLC isolated A1PI N-glycans

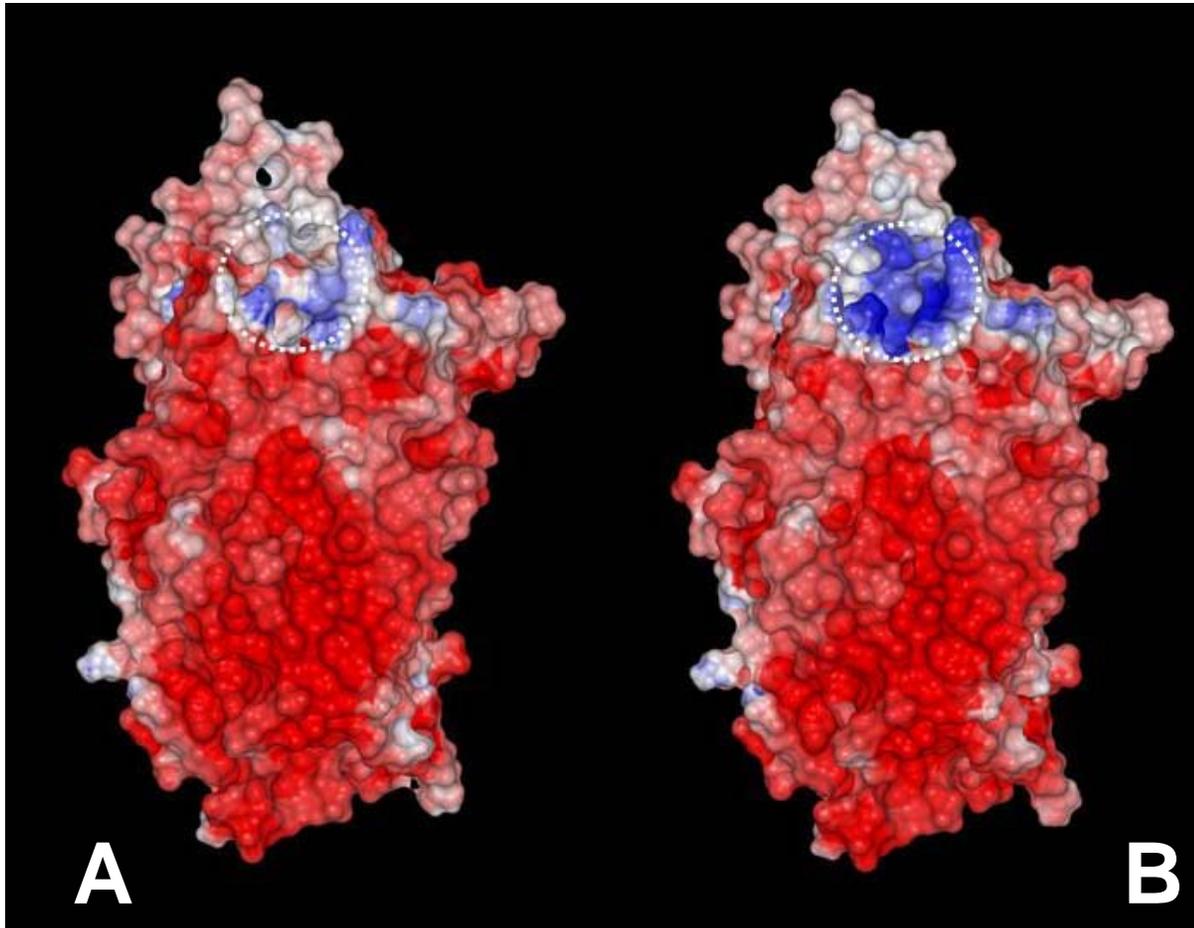
Isolated A1PI N-glycans are labelled with a fluorescent dye

Different structural isomers can be separated and characterized by HPLC retention time before and after treatment with specific exoglycosidases



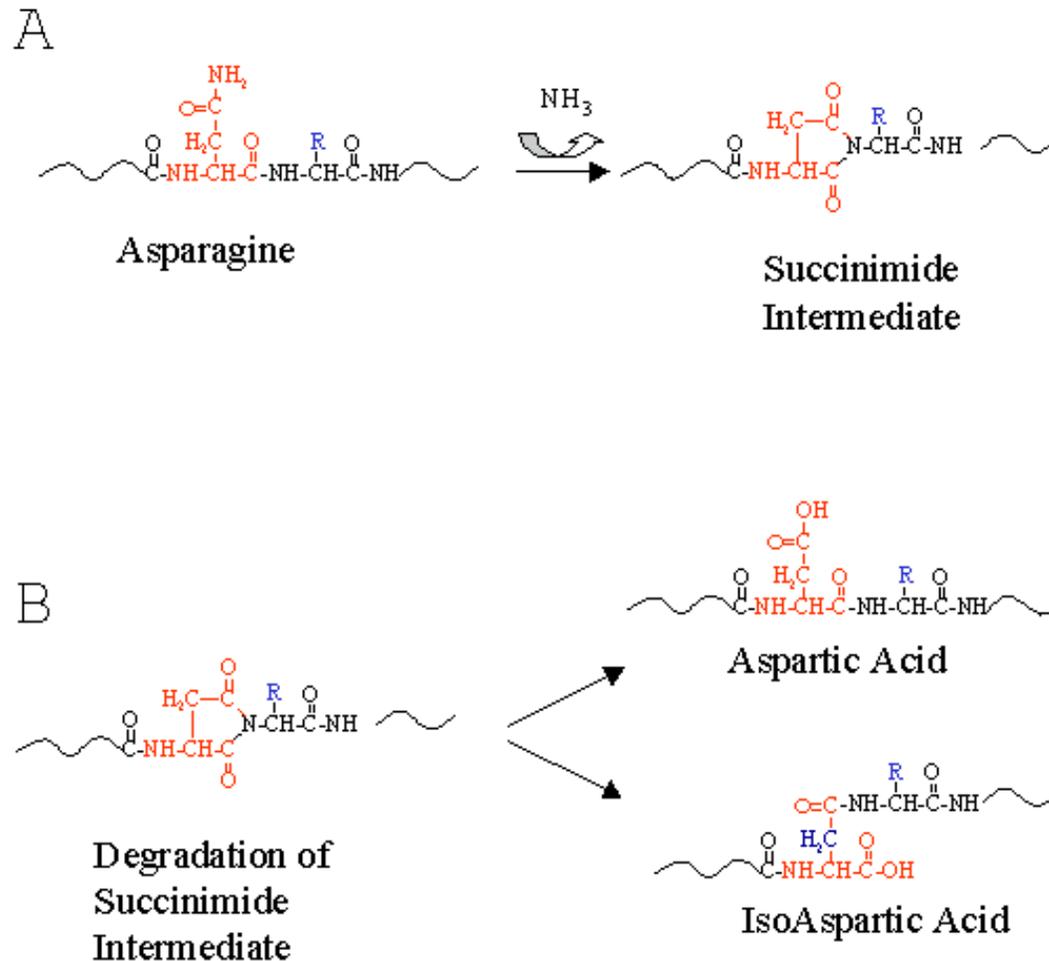
* Other for glycan analysis widely used fluorophores are e.g. 2-aminobenzamide (AB)

Cysteinylation of Cys-232: Electrostatic Surface Potential Maps



(A) cysteinylated cys-232; (B) free cys-232

Deamidation of Asparagine in Proteins



Carboxypeptidase N, Ethanol Fractionation and Aralast

Carboxypeptidase N (CPN) is the most likely candidate causing the IEF pattern of Aralast:

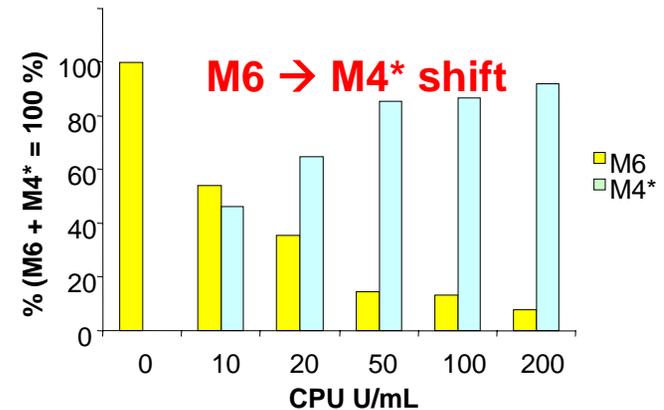
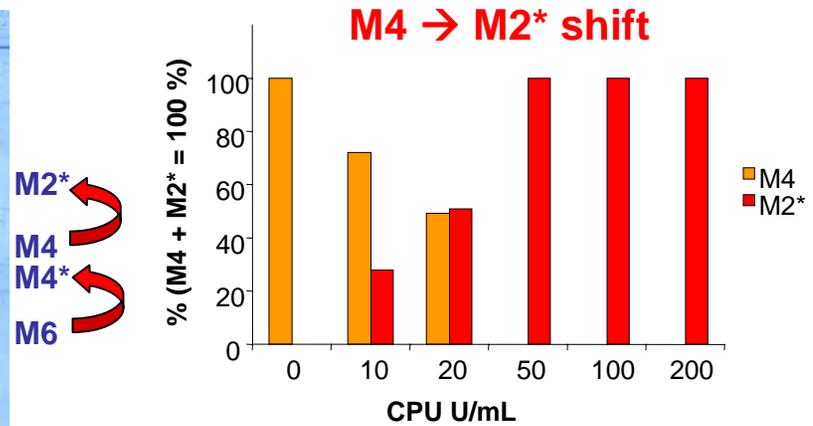
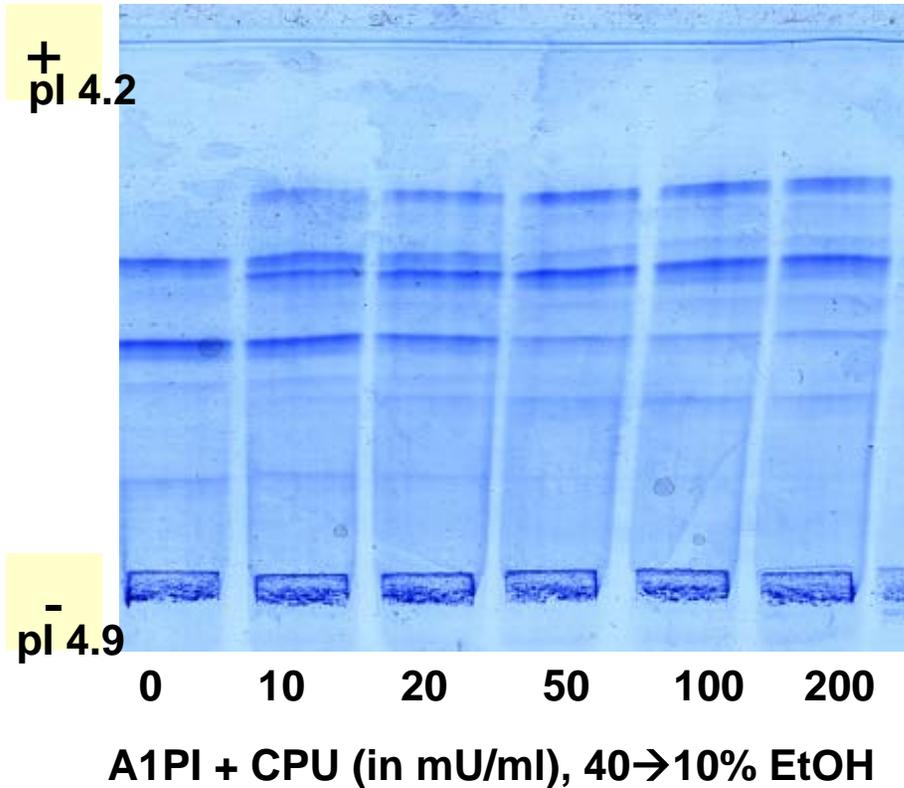
Plasma does not contain CPU (only proCPU or TAFI)

Trace amounts of of plasmin/thrombin/thrombomodulin could activate some CPU

Experiments with the basic carboxypeptidase inhibitor Mergetpa support CPN; however, Mergetpa also inhibits CPU, but at a lower potency ($K_i=2\text{nM}$ for CPN and 750 for CPU)

Mergetpa = DL-2-mercapto-methyl-3-guanidino-ethylthiopropionic acid

A1PI Isoform Shift Induced by Treatment with CPU (TAF1a)



A1PI from IV,1

Enzyme: CPU (0.5 U/ml)

A1PI (+4°C, 2mg/ml) + 96% EtOH (-20°C), 20 min at -20°C;

+ 10 mM TRIS/HCl, pH 8.8 (+4°C) + CPU (4°C); incubated for 60 min at +37°C



Microscopic distribution of Aralast

CENTRAL AIRWAY

