Characterization of a New Alpha₁-proteinase Inhibitor from Human Plasma—Prolastin®-C

Todd Willis, PhD,¹ Kevin Wee, PhD,² Koen van der Drift, PhD² and Gerold Mohn, PhD²


Abstract
Scientific advances have enabled modifications of the manufacturing process of Prolastin® (human alpha₁-proteinase inhibitor) to significantly increase active content and functional activity. The objective of this study was to determine the biochemical characteristics of the new product, Prolastin®-C, including potency, purity, and glycan profile. Prolastin-C was characterized using a panel of analytical methods consisting of anti-neutrophil elastase capacity, immunonephelometry, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and capillary gel electrophoresis (SDS-CGE), capillary zone electrophoresis, Western blotting, isoelectric focusing, size exclusion high-performance liquid chromatography, and mass spectrometry. Six lots of Prolastin-C were characterized. These had a mean functional activity of 54.3 mg/ml (approximately twice that of Prolastin). The average purity of Prolastin-C was 97% and the monomeric content was 89%. The glycan profile was comparable to that found in normal human plasma, while mass spectrometry confirmed the expected mass and presence of the single cysteine in Prolastin-C. Five plasma protein impurities could be quantified, accounting for approximately 4% of the total protein content.

Keywords
Alpha₁-antitrypsin, alpha₁-proteinase inhibitor, Prolastin, Prolastin-C, functional activity, purity, potency, glycan profile

Materials and Methods
The following analytical methods were used to evaluate six final container lots of Prolastin-C manufactured at commercial scale.

Potency
The functional activity of alpha₁-PI was determined by measuring antineutrophil elastase capacity. The rate of the elastase activity reaction, measured with a chromogenic substrate (Suc-(Ala)₃-pNA), is
Alpha₁-antitrypsin Deficiency

Table 1: Functional Activity of Prolastin-C (Mean ± 1 Standard Deviation)

<table>
<thead>
<tr>
<th>Test</th>
<th>Prolastin-C (n = 6 lots)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Functional activity (mg/ml)*</td>
<td>54.3 ± 3.1</td>
</tr>
<tr>
<td>Total protein content (mg/ml)</td>
<td>55.2 ± 1.7</td>
</tr>
<tr>
<td>Alpha₁-PI antigen content (mg/ml)</td>
<td>52.1 ± 3.5</td>
</tr>
<tr>
<td>Specific activity (functional activity/protein content)</td>
<td>0.98 ± 0.03</td>
</tr>
<tr>
<td>Active content (functional activity/antigen content)</td>
<td>1.04 ± 0.04</td>
</tr>
</tbody>
</table>

*Anti-neutrophil elastase capacity.

Figure 1: SDS-CGE (A) and SDS-PAGE (B) of Prolastin-C under Non-reducing Conditions

The bands appearing above and below the prominent alpha₁, band in SDS-CGE (A) represent the internal standards used in the SDS-CGE method.

SDS-CGE = sodium dodecyl sulfate capillary gel electrophoresis.

Reversed-phase HPLC (RP-HPLC) was performed using an Agilent 1100 system and a 2.1mm x 150mm Waters Symmetry® C4 column. A gradient elution using 0.1% trifluoroacetic acid in water and increasing amounts of 0.1% trifluoroacetic acid in acetonitrile were used, with detection at 214nm. The column eluate was subjected to electrospray mass spectrometry using an Agilent LC/MSD single quad mass spectrometer. Spectra deconvolution to assess the glycosylation heterogeneity was carried out using MassLynx (Waters) software.

Matrix-assisted laser desorption ionization (MALDI) was performed on the Voyager DE STR (Applied Biosystems). Samples analyzed by MALDI mass spectrometry were cleaned up using C₄ ZipTips (Millipore). Approximately 0.5µl of a saturated sinapinic acid solution in 50% acetonitrile (ACN) was spotted on the MALDI plate followed by 0.5µl of the cleaned protein solution. Spectra were recorded in the linear mode, using bovine serum albumin (BSA) as external calibrant.

Glycan Profile
Isoelectric focusing (IEF) was performed using a pre-cast Novex® IEF gel (Invitrogen Corporation) with an ampholyte range of pH 3–7. Gels were either stained with SimplyBlue dye or used for Western blotting. Western blots were performed using a SNAP i.d.™ system (Millipore) with an antialpha₁-PI monoclonal primary antibody (QED Bioscience) and a Thermo ImmunoPure Goat AntiMouse Immunoglobulin G (IgG) (H+L) (Thermo Fisher Scientific) secondary antibody. The Western blot was stained using bromo-chloro-indolyl phosphate/nitroblue tetrazolium substrate.

Impurities
Plasma protein impurities were assessed by immunonephelometry using a Dade Behring BN II Nephelometer. The 16 proteins tested were albumin, alpha₂ macroglobulin, alpha₁ acid glycoprotein, apolipoprotein A-1, apolipoprotein B, antithrombin III, ceruloplasmin, fibrinogen, fibrinectin, haptoglobin, IgA, IgG, IgM, plasminogen/plasmin, prealbumin, and transferrin.

Results Potency
The mean functional activity of six final container lots of Prolastin-C manufactured at commercial scale was 54.3mg/ml (see Table 1), representing approximately twice the functional activity of Prolastin at approximately 25mg/ml. The higher functional activity of Prolastin-C was associated with a mean specific activity of 0.98mg/mg and active content of 1.04mg/mg (see Table 1).
Six different lots of Prolastin-C were evaluated by CZE. The mean alpha\textsubscript{1}-PI purity by CZE was measured at 97%. SDS-PAGE and SDS-CGE showed similar purity levels with a prominent alpha\textsubscript{1}-PI band at approximately 50kDa and 29 seconds, respectively (see Figure 1). Under non-reducing conditions, SDS-PAGE showed a faint band with a molecular weight of approximately 100kDa. Based on its molecular weight and detection with an antihuman alpha\textsubscript{1}-PI antibody probe in Western blots, the faint band represents a low level of alpha\textsubscript{1}-PI dimer. SE-HPLC separated Prolastin-C into monomer (50kDa), oligomer (100–500kDa), and high-molecular-weight aggregate (>500kDa) regions (see Figure 2). The molecular masses of these three regions were defined by light scattering. The six Prolastin-C lots showed an average monomer level of 88.8% and contained a relatively low aggregate level of ≤1% (see Table 2).

Mass spectrometry using MALDI confirmed the purity of Prolastin-C by not detecting the presence of other plasma proteins. The reported molecular weight of m/z 50001 for alpha\textsubscript{1}-PI was an average of all isoforms.

### Glycan Profile

IEF of Prolastin-C revealed the two primary isoforms of alpha\textsubscript{1}-PI (M6 and M4), together with a faint band representing the minor isoform (M2). This profile of Prolastin-C is comparable to that found in normal human plasma, as confirmed by Western blotting using an anti-alpha\textsubscript{1}-PI antibody probe (see Figure 3).

**Impurities**

Deconvolution of the electrospray mass spectrometry data collected after RP-HPLC of Prolastin-C showed a closely spaced pattern of molecular masses representing the multiple glycoforms of alpha\textsubscript{1}-PI (see Figure 4). The primary glycoforms of alpha\textsubscript{1}-PI are M6 and M4, with the higher-mass species likely representing the naturally-occurring fucosylated glycoforms of M6 and M4 (M6 + fucose, M4 + fucose).

The presence of the naturally-occurring single cysteine in Prolastin-C was proven by intact protein mass spectrometry (using electrospray ionization) after processing under reducing and non-reducing conditions. The reduced profile is shifted to the left compared with the non-reduced profile. This reflects the decrease in

### Table 2: Molecular Distribution of Prolastin-C by Size Exclusion High-performance Liquid Chromatography (Mean±1 SD)

<table>
<thead>
<tr>
<th>Test</th>
<th>Prolastin-C (n = 6 lots)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer (%)</td>
<td>88.8 ± 1.7</td>
</tr>
<tr>
<td>Average monomer mass</td>
<td>~50kDa</td>
</tr>
<tr>
<td>Oligomer (%)</td>
<td>10.5 ± 1.6</td>
</tr>
<tr>
<td>Oligomer mass range</td>
<td>100–500kDa</td>
</tr>
<tr>
<td>Aggregate (%)</td>
<td>≤1.0</td>
</tr>
<tr>
<td>Aggregate mass range</td>
<td>&gt;500kDa</td>
</tr>
</tbody>
</table>

Purity

Six different lots of Prolastin-C were evaluated by CZE. The mean alpha\textsubscript{1}-PI purity by CZE was measured at 97%. SDS-PAGE and SDS-CGE showed similar purity levels with a prominent alpha\textsubscript{1}-PI band at approximately 50kDa and 29 seconds, respectively (see Figure 1). Under non-reducing conditions, SDS-PAGE showed a faint band with a molecular weight of approximately 100kDa. Based on its molecular weight and detection with an antihuman alpha\textsubscript{1}-PI antibody probe in Western blots, the faint band represents a low level of alpha\textsubscript{1}-PI dimer. SE-HPLC separated Prolastin-C into monomer (50kDa), oligomer (100–500kDa), and high-molecular-weight aggregate (>500kDa) regions (see Figure 2). The molecular masses of these three regions were defined by light scattering. The six Prolastin-C lots showed an average monomer level of 88.8% and contained a relatively low aggregate level of ≤1% (see Table 2).
Alpha\(_1\)-antitrypsin Deficiency

molecular weight associated with loss of the additional free cysteine linked by cysteinylation to the single cysteine in alpha\(_1\)-PI.

Measurement of specific plasma protein impurities in Prolastin-C by immunonephelometry detected only low levels of albumin (0.5%), alpha-1 acid glycoprotein (0.38%), apolipoprotein A-I (0.033%), haptoglobin (0.92%), and IgA (2.4%). On average, these five protein impurities accounted for approximately 4% of the total protein content, with alpha\(_1\)-PI comprising 96% of the total protein content. Eleven other potential plasma protein impurities evaluated by immunonephelometry were below their limits of detection.

Discussion

Recent improvements to the Prolastin manufacturing process have led to the production of Prolastin-C, a human alpha\(_1\)-PI product with a functional activity approximately twice that of Prolastin. The increase in functional activity of Prolastin-C was accomplished through process improvements that decreased plasma protein impurities and increased alpha\(_1\)-PI-specific activity. Ultimately, the higher functional activity of Prolastin-C improves patient convenience by significantly reducing the time required to administer the product. Infusion times with Prolastin-C are approximately 15 minutes.

Protein purification processes can have subtle effects on the integrity of any protein extracted. This is evident from the differences previously identified between the various alpha\(_1\)-PI products.\(^3\) In normal human plasma (and Prolastin), Cys232 of alpha\(_1\)-PI is cysteinylated (the cysteine is linked to an additional cysteine amino acid).\(^2\) This may influence the interaction of the protein with its physiological ligands.\(^2\) It is also important to retain this cysteinylation in the product because it minimizes the formation of a protein dimer linked through a disulfide bond. In Prolastin-C, this cysteinylated cysteine was shown to be present by mass spectrometry following RP-HPLC (see Figure 4), as is the case in normal plasma.

The glycan profile of Prolastin-C was comparable to naturally-occurring alpha\(_1\)-PI in normal human plasma, which exists as an acidic glycoprotein. In plasma, alpha\(_1\)-PI has some heterogeneity due to post-translational modifications, principally in the three complex N-glycans that are covalently linked to three different asparagine residues.\(^3\) These N-glycans have different numbers of sialic acid residues, conferring slightly different levels of negative charge that can be detected using IEF.\(^2\) There are two primary glycoforms (M4 and M6) and other minor glycoforms (which include M2). Correct glycosylation of glycoproteins is important, as it can affect protein folding,\(^{11}\) biologic activity,\(^{12}\) specific interactions with receptors\(^{13,14}\) and half-life.\(^{15}\) In this study, the IEF profile of Prolastin-C was comparable to that of normal human plasma, indicating that the modified purification process does not appreciably alter the normal glycoform composition.

Conclusion

In conclusion, this study shows that the alpha\(_1\)-PI in Prolastin-C is of a higher potency compared with Prolastin, while maintaining excellent purity and the same physical and chemical characteristics.

---