Study of Hydrodynamic Properties of α-1-antitrypsin from Mammalian Serum

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Abstract. Alpha-1-antitrypsin is an acute phase protein which is up-regulated in acute phase responses to tissue necrosis and inflammation. It belongs to a class of inhibitors called “Serpins” (serine protease inhibitors). Human plasma contains a number of proteinase inhibitors. Among these, α-1-antitrypsin is found in the highest concentration, and is the major inhibitor for neutrophil elastase. Not only does α-1-antitrypsin deficiency lead to the disabling syndrome of pulmonary emphysema, there are other disorders too which include ANCA (antineutrophilic cytoplasmic antibody) positive Wegener’s granulomatosis, diffuse bronchiectasis, necrotizing panniculitis in α-1-antitrypsin phenotype (S), idiopathic pulmonary fibrosis and steroid dependent asthma. In view of its varied important roles in humans, a mammalian source was chosen for isolation, purification and studies into the hydrodynamic properties performed on the homogeneous fraction. Hydrodynamic properties of buffalo serum α-1-antitrypsin were determined by gel filtration, which gave a value of 3.23 nm for the stokes radius of the protein. The diffusion coefficient was found to be 6.85 x 10^-7 cm^2 s^-1 and the frictional ratio was found to be 1.27, which together suggested a slightly aglobular conformation and excessive hydration of the isolated inhibitor molecules.

Keywords: α-1-antitrypsin, hydrodynamic properties, mammalian serum.

INTRODUCTION

Alpha-1-antitrypsin is an acute phase protein which means that production by the liver is subject to various stimuli including inflammatory mediators induced by fever. It is the main proteinase inhibitor (Pi) in human serum (Fregonese & Stolk, 2008; agerhol and Laurell, 1967). Alpha-1-antitrypsin deficiency (AATD) is a genetic disorder that manifests as pulmonary emphysema, liver cirrhosis and rarely as the skin disease panniculitis (Fregonese & Stolk, 2008; agerhol and Laurell, 1967; World Health Organisation, 1997; de Serres, 2002). The protein alpha-1-antitrypsin is produced in hepatocytes and released into the circulating blood by the liver (Brantly et al., 1988a). The genetic defect in α-1-antitrypsin (AAT) deficiency alters the configuration of the α-1-antitrypsin molecule and prevents its release from hepatocytes. As a result, serum levels of α-1-antitrypsin are decreased, leading to low alveolar concentrations, where the α-1-antitrypsin molecule normally would serve as protection against anti proteases. The resulting protease excess in alveoli destroys alveolar walls and causes emphysema. The accumulation of excess α-1-antitrypsin in hepatocytes can also lead to destruction of these cells and, ultimately, clinical liver disease (Campos et al., 2003; Fairbanks and Tavill, 2008; Petrache et al., 2006). Though present in all body tissues, AAT has its primary physiological significance in the lungs, where it protects the healthy but fragile alveolar tissue from proteolytic damage by enzymes like neutrophil elastase (Gadek et al., 2003). Cigarette smoking accelerates the progression of emphysema in patients with α-1-antitrypsin deficiency (Brantly et al., 1988b). It was also found that during uncontrolled diabetes there is reduced trypsin inhibitory capacity due to glycosylation of α-1-antitrypsin (Naderi et al., 2006). The production of α-1-antitrypsin is controlled by a pair of genes at the protease inhibitor (Pi) locus. The SERPINA1 (formerly known as Pi) gene responsible for encoding α-1-antitrypsin is located on chromosome 14 and is highly pleomorphic, with more than 100 allelic variants. The most common form of α-1-antitrypsin deficiency is associated with allele Z in the homozygous state (ZZ). Other genotypes associated with severe AATD include PiSZ, PiZ/Null and PiNull (Naderi et al, 2006; ATSERS, 2003; Cox & Levison, 1988; Eriksson, 1964; Kramps et al., 1980; Turino et al., 1996; Clark et al., 1982; Yamamoto et al., 1986; Kalsheker et al., 2002). McNab et al. (2007) showed that the defective gene in α-1-antitrypsin deficient individuals can be corrected by transfection of small DNA fragments of M type α-1-antitrypsin that increases the production of the protein.

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Serine proteases have been isolated from plants as well as microbes (McNab et al., 2007; George-Okafor & Odibo, 2011). Infusion of purified AAT from pooled human plasma, so-called "augmentation therapy", represents a specific therapy for AAT deficiency (Stoller & Aboussouan, 2012; Miravitlles, 2012). In view of its varied important role in humans, α-1-antitrypsin also appears to be antibacterial and an inhibitor of viral infections, such as influenza and HIV, and is currently being evaluated in clinical trials for Type-1 diabetes, cystic fibrosis and graft-versus-host-disease (Lewis, 2012) and its role in therapy (Stoller & Aboussouan, 2012; Miravitlles, 2012). For this current study a mammalian source was chosen for the isolation and purification, and for studies into the hydrodynamic properties of the protein were performed on the homogeneous fraction. Hydrodynamic properties of buffalo serum α-1-antitrypsin were determined by gel filtration on Sephacryl S-200HR and can be compared at some later stage with human α-1-antitrypsin, potentially to be used as "augmentation therapy".

MATERIALS AND METHODS

Column Chromatographic Media Sephacryl S-200 HR and blue dextran-2000 were obtained from Sigma Chemical Company, USA. DEAE-Sephacel with a bead size of 40-60µ having a capacity of 100-140 eqv./ml of gel volume was also obtained from Sigma Chemical Company, USA.

Ion-Exchange Chromatography Preswollen DEAE-Sephacel suspension in 20% ethanol was suspended in distilled water and fine particles were removed by repeated decantation. The gel slurry was poured into the column and the gel bed was stabilized by a 0.03M sodium phosphate buffer pH 6.8, Is = 0.042, with the same pH and ionic strength as that of the sample. For sample application the column was drained of the buffer and the sample were applied. The unbound sample was eluted with 0.03M sodium phosphate buffer pH 6.8, Is = 0.042, and then in a batch-wise manner, using buffers of different ionic strengths (Is = 0.042, 0.1, 0.2, 0.5 and 1.0) fractions of 2-3ml were collected and monitored by the method of Lowry et al. (1951).

Gel-Filtration Preswollen Sephacryl S-200HR was packed in a glass column (63 x 1.61cm). The radius of the column was determined at three different places of the column. The volume of water (V) corresponding to a 3 cm height in the column (h) was determined by dividing the weight (w) of water by its density (d) at a particular temperature, and the radius of the column was calculated as follows:

\[ V = \pi r^2 h \]
\[ \pi r^2 h = w/d \]

The total volume of the column was calculated by the following equation:

\[ V_t = \pi r^2 h \]

where h denotes the total volume of the column.

The gel bed was stabilized by sodium phosphate buffer, pH=7.0, Is = 0.15.

The elution volume (Ve) of the blue dextran yielded the void volume (Vo) of the column. The internal volume (Vi) of the column was obtained by subtraction of the void volume from the elution volume of glucose (Miravitlles, 2012). The Sephacryl S-200HR column was calibrated by elution of marker proteins of known hydrodynamic properties. The eluted samples were collected in 2-5 ml fractions and monitored by the method of Lowry et al. (1951).

Results and Discussion

Hydrodynamic properties of buffalo serum α-1-antitrypsin were determined by analytical gel chromatography on Sephacryl S-200HR column equilibrated in 0.06M sodium phosphate buffer, pH 7.0, Is = 0.15.

The void volume (Vo) of the column was determined by passing a band of blue dextran, the inner volume was determined by passing 0.5% glucose solution, and the elution is given by the equation:

\[ V_{o,d} = V_o + K_d V_i \]  \hspace{1cm} (1)

The value of the distribution coefficient, Kd for glucose was 112. Using these values Vf was calculated to be 63 ml. Kf is calculated for a given molecular type and represents the fraction of the stationary phase that is available for the substance. In practice Kf is difficult to determine and it is usually replaced by Kf’.

\[ K_d = (V_e - V_f) / V_f \] \hspace{1cm} (2)
\[ K_n = (V_e - V_n) / (V_e - V_f) \] \hspace{1cm} (3)

The total volume of the column was determined to be 124 ml. The marker proteins used in gel filtration along with their Stokes radii and molecular weights are given in Table 1.

The elution profile of various marker proteins [BSA (dimer and monomer), ovalbumin, α-chymotrypsinogen and...
Figure 1. Elution profile of marker proteins and buffalo serum alpha-1 antitrypsin on Sephacryl S 200 HR columns (63 x 1.61 cm). Marker proteins used were (a) BSA (dimer) and BSA (monomer), (b) Ovalbumin, (c) α-Chymotrypsinogen, (d) Cytochrome c, and (e) Buffalo serum alpha-1-antitrypsin.

Table 1. Values of molecular weights and stokes radii of marker proteins. Adapted from Andrews (1970), and Tanford (1968).

<table>
<thead>
<tr>
<th>Marker proteins</th>
<th>Molecular weight</th>
<th>Stokes radius (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA (dimer)</td>
<td>132</td>
<td>4.3</td>
</tr>
<tr>
<td>BSA (monomer)</td>
<td>68</td>
<td>3.5</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>45</td>
<td>3.0</td>
</tr>
<tr>
<td>α-chymotrypsinogen</td>
<td>25</td>
<td>2.29</td>
</tr>
<tr>
<td>Cytocrome C</td>
<td>12</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Table 2. V/Vo, Log M, Kd 1/3 and M 1/3 values for marker proteins and buffalo serum. Each value in the table represents four determinations carried out in triplicate, and in no case was the difference found to be more than six percent.

<table>
<thead>
<tr>
<th>Marker proteins</th>
<th>V/Vo</th>
<th>Log M</th>
<th>Kd 1/3</th>
<th>M 1/3</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA (dimer)</td>
<td>1.24</td>
<td>5.12</td>
<td>0.57</td>
<td>51.29</td>
</tr>
<tr>
<td>BSA (monomer)</td>
<td>1.33</td>
<td>4.83</td>
<td>0.65</td>
<td>40.8</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>1.43</td>
<td>4.65</td>
<td>0.70</td>
<td>35.5</td>
</tr>
<tr>
<td>α-chymotrypsinogen</td>
<td>1.63</td>
<td>4.40</td>
<td>0.79</td>
<td>29.5</td>
</tr>
<tr>
<td>Cytocrome C</td>
<td>1.93</td>
<td>4.09</td>
<td>0.98</td>
<td>22.7</td>
</tr>
<tr>
<td>α-1-antitrypsin</td>
<td>1.37</td>
<td>0.67</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Kd, Kav, erfc-1Kd and (-log Kav)1/2 values of marker proteins and buffalo serum α-1-antitrypsin. Each value in the table represents an average of 4 determinations carried out in duplicate, and in no case was the difference found to be more than three percent.

<table>
<thead>
<tr>
<th>Proteins/sample</th>
<th>Kd</th>
<th>Kav</th>
<th>erfc-1Kd</th>
<th>(-log Kav)1/2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA (dimer)</td>
<td>0.19</td>
<td>0.16</td>
<td>0.94</td>
<td>0.8921</td>
</tr>
<tr>
<td>BSA (monomer)</td>
<td>0.27</td>
<td>0.22</td>
<td>0.80</td>
<td>0.8109</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>0.33</td>
<td>0.28</td>
<td>0.72</td>
<td>0.7435</td>
</tr>
<tr>
<td>α-chymotrypsinogen</td>
<td>0.49</td>
<td>0.41</td>
<td>0.62</td>
<td>0.6222</td>
</tr>
<tr>
<td>Cytocrome C</td>
<td>0.79</td>
<td>0.61</td>
<td>0.45</td>
<td>0.4632</td>
</tr>
<tr>
<td>α-1-antitrypsin</td>
<td>0.30</td>
<td>0.25</td>
<td>0.74</td>
<td>0.7701</td>
</tr>
</tbody>
</table>

cytochrome c] and chymotrypsinogen α-1-antitrypsin are depicted in Figure 1. The elution volume of α-1-antitrypsin was calculated to be 67 ml from the average of three readings.

The following equations were used for the analysis of gel filtration data by the method of least square to determine the molecular weight:

\[ V/V_o = 0.205 \log M + 0.422 \]
\[ M^{1/3} = 64.27 K_d^{1/3} + 82.36 \]

where \( M \) is the molecular weight of α-1-antitrypsin. The linear plots according to equations 4 and 5 are depicted in Figures 2 and 3. The values of \( V/V_o \), \( \log M \) and \( K_d^{1/3} \) and \( M^{1/3} \) for marker proteins and α-1-antitrypsin are given in Table 2.

The value of \( V/V_o \) of 1.37 for α-1-antitrypsin corresponds, according to Figure 2, to a molecular weight of 53 kDa. From the linear plot shown in Figure 3, the molecular weight was found to be 59.8 kDa, using the \( K_d^{1/3} \) value for α-1-antitrypsin of 0.67. The average of the values gave a molecular weight of 56.4 kDa for buffalo serum α-1-antitrypsin. The value (56.4 kDa) obtained by gel filtration is higher than the value brained during SDS-PAGE (51 kDa). The values of \( K_o, K_w, \text{erfc}^{-1} K_d \), and \((-\log K_w)^{1/2}\) were calculated for marker proteins and α-1-antitrypsin and the results are summarized in Table 3.

The stokes radius of α-1-antitrypsin was determined by analyzing the gel filtration data according to the method of Ackers (1967) using the following equation:

\[ \text{erfc}^{-1} K_d = 0.19 (r) + 0.13 \]

Figure 4. Analysis of gel filtration data of marker proteins and buffalo serum alpha-1-antitrypsin, according to the method of Ackers (1967). Marker proteins used were as follows BSA (dimer), BSA (monomer), Ovalbumin, α-Chymotrypsinogen, and Cytochrome c.

where erfc⁻¹Kd is the error function complement of distribution factor of the protein with the stokes radius (r). Least square analysis of plot erfc⁻¹Kd versus r yielded a straight line (Figure 4). The value of erfc⁻¹Kd for α-1-antichymotrypsin was calculated to be 0.74, which according to equation (6) would correspond to stokes radius (r) of 3.210 nm.

Another plot was drawn according to Laurent and Killander 27 between stokes radius (r) and (-logKav)¹/² as shown in Figure 5. A straight line was obtained by a least square analysis which fits the equation:

\[ (-\log K_{av})^{1/2} = 0.019 (r) + 0.708 \]  (7)

The value of (-logKav)¹/² for α-1-antitrypsin was found to be 0.770, which according to equation (7) corresponds to a stokes radius (r) value of 3.26 nm.

From the knowledge of the stokes radius (r), the diffusion coefficient, D, of α-1-antitrypsin was calculated according to the equation:

\[ D = kT/6\pi\eta r \]  (8)

where K is the Boltzmann constant, \( \eta \) is the coefficient of viscosity of the medium (i.e. 0.06M sodium phosphate buffer, pH 7.0, Is = 0.15) and T is the absolute temperature. Using values of K= 1.386 x 10⁵ ergs/deg, \( \eta = 0.01 \) poise, T= 298 K and from the determined value of r. The value of the diffusion coefficient for α-1-antitrypsin was calculated to be 6.85 x 10⁻⁷ cm²/sec from the above equation (8).

The frictional ratio \( f/f_0 \) of the α-1-antitrypsin was calculated using the relation shown below (Laurent & Killander, 1964):

\[ f/f_0 = r \left( \frac{3\eta}{4\pi N} \right)^{1/3} \]  (9)

when N is Avogadro’s number (6.023 x 10²³ per mole) and \( \eta \) is the partial specific volume of the protein. The partial specific volume of buffalo serum α-1-antitrypsin was taken to be 0.728 as that reported for human α-1-antitrypsin (Andrew, 2006). The molecular weight of buffalo serum α-1-antitrypsin was taken to be 56.4 kDa as determined by gel filtration. Using these values the frictional ratio of α-1-antitrypsin was calculated to be 1.270 with the help of equation (9).

CONCLUSION

The gel filtration analysis of buffalo serum α-1-antitrypsin on a Sephacryl S-200HR column gave the value of stokes radius of the inhibitor to be 3.230 nm and the molecular weight to be 56.4 kDa. The value of molecular weight (MW) obtained by gel filtration was found to be 9% higher than the value obtained by SDS-PAGE. The higher value of MW by gel filtration was expected as the inhibitor contained about 13.5% carbohydrate. The diffusion coefficient of the buffalo serum α-1-antitrypsin was found to be 6.85 x 10⁻⁷ cm² sec⁻¹ for which the frictional ratio (f/f₀) was found to be 1.270. The values of D and f/f₀ determined for the inhibitor taken together suggest that the native inhibitor molecule has a non-globular conformation. Further, the deviation of f/f₀ value from unity may be attributed to excessive hydration of the inhibitor. This explanation appears to be a real possibility since buffalo serum α-1-antitrypsin, being a sialoglycoprotein containing about 13.5% carbohydrate, must be substantially hydrated.

This study suggests that the buffalo serum α-1-antitrypsin has characteristics close to ovine, dog, sheep and more importantly to human α-1-antitrypsin in terms of its hydrodynamic properties such as molecular weight, carbohydrate content (Gupta et al., 2008; Mistry et al., 1991; Malgarejo et al., 1996). Since it has already been established that infusion of purified AAT from pooled human plasma, so-called “augmentation therapy”, represents a specific therapy for AAT deficiency (Stoller & Aboussouan, 2012; Miravitlles, 2012). The similarities in the hydrodynamic properties of buffalo serum α-1-antitrypsin with other sources of mammalian α-1-antitrypsin mean that it can be further studied and be a potential source for “augmentation therapy”, as this
approach raises serum levels above the protective threshold. It also suggests that this approach would be safe and could slow the decline of lung function and emphysema progression and can also be used for uncommon AAT deficiency-related diseases other than pulmonary emphysema, such as fibromyalgia, systemic vasculitis, relapsing panniculitis and bronchial asthma (Blanco et al., 2011).

Other parameters like the amino acid sequence, the effect of denaturants, and the thermolability or thermostability of the inhibitor will be the interesting basis of future studies.

REFERENCES


hydrodynamic properties of α-1-antitrypsin


