Chromatography
Separation Techniques

Kumar et al., J Chromatogr Sep Tech 2015, 6:4
DOI: 10.4172/2157-7064.1000277

Research Article Open Access

Purification of A1PI from Human Plasma-An Improved Process to Achieve Therapeutic Grade Purity

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Abstract

Alpha-1-proteinase inhibitor (A1PI) also known as α-anti trypsin (AAT) is a member of serine protease inhibitors super family present in human plasma. A1PI is the only known inhibitor of elastase but also inhibits trypsin and chymotrypsin. A1PI is a molecule with lot of therapeutic importance and is widely used in the treatment of emphysema or chronic obstructive pulmonary disease (COPD). There are many methods detailed in scientific articles and patents to purify A1PI but the current research describes a simple three step chromatography procedure to fractionate A1PI directly from human plasma. The current purification process starts from human plasma unlike most of the existing methods where Cohn fraction IV-1 paste is the starting material. This method excludes the use of more methods like Cohn ethanol fractionation, precipitation or affinity capture. The A1PI purified by this scheme is characterized and found to be a single chain glycoprotein with a molecular weight of 52 KDa with increased purity. As the process is devoid of precipitation and affinity steps, this scheme is easily scalable, economical to adapt for manufacturing A1PI with good yields of 0.6-0.7 gm of A1PI/L of plasma.

Keywords: Alpha-1-proteinase inhibitor; DEAE sepharose; Fractogel TMAE; Capto adhere

Abbreviations: A1PI: Alpha-1-Proteinase Inhibitor; PEG: Poly Ethylene Glycol; DEAE: Diethyl Amino Ethyl; TMAE: Tri Methyl Amino Ethyl; BAPA: Benzoyl-DL-Arginine-p-Nitroanilide.

Introduction

Human Alpha-1-proteinase inhibitor (A1PI) is a well characterized multifunctional protease inhibitor, also known as α-antitrypsin (AAT). It is a glycoprotein with molecular weight of about 52000 Daltons and is a single polypeptide chain to which several oligosaccharide units are covalently bound. It belongs to the serine protease inhibitor (serpin) super family, which in addition to A1PI also includes α1-antichymotrypsin, antithrombin, plasminogen activator inhibitor, C1 esterase inhibitor [1,2]. A1PI is comprised of 394 amino acid residues, including one cysteine, 2 tryptophans and 9 methionine residues [3,4]. Three N-linked glycans attached to asparagine residues 46, 83, and 247 represent ~12% of A1PI by molecular weight [5,6,7]. It synthesized in the liver and is present in the serum at level between 150 to 350 mg/dl (30-80 μM) when assayed with plasma standard. The major physiological role of A1PI is to inhibit neutrophil elastase (NE) in the lungs. A1PI is an inhibitor of proteases such as trypsin, chymotrypsin, pancreatic elastase, skin collagenase, rennin, urokinase and protease of polymorphonuclear lymphocytes. Alpha-1-proteinase inhibitor deficiency is an autosomal co-dominant, hereditary disorder characterized by low serum and lung levels of alpha1-PI. Severe forms of the deficiency are frequently associated with progressive, moderate to severe emphysema that may become apparent in adults at any age regardless of smoking history, potentially resulting in a lower life expectancy. In the absence of A1PI, neutrophil elastase, released by lung macrophages and neutrophils, damages protein components of the alveolar wall leading to elastin breakdown and the loss of lung elasticity and lung tissue. Excessive elastolytic activity underlies progressive emphysema in patients with low plasma A1PI levels (<11 μM). A1PI augmentation therapy specifically aims to restore the protease-antiprotease balance in lung tissue of such patients and to attenuate the progression of pulmonary emphysema. Based on the A1PI serum concentration, a common classification to define A1PI deficiency includes the four major categories: (1) normal (with A1PI serum levels not lower than 20 μM); (2) deficient (with A1PI concentrations in serum lower than 20 μM); (3) dysfunctional (with normal A1PI level, but lost or lower inhibitory activity); and (4) null (with A1PI serum concentrations below the detectable level) [8]. Currently, A1PI therapeutic preparations are licensed exclusively for one indication, i.e. chronic augmentation and maintenance therapy in individuals with emphysema due to congenital A1PI deficiency but as evident from the available literature, due to the multiple biological activities of A1PI, it has been associated with other lung diseases (cystic fibrosis) and many non-pulmonary diseases like vasculitis, fibromyalgia, asthma, pancreatitis, rheumatoid arthritis, atherosclerosis [8]. Currently, there are four US Food and Drug Administration (US FDA) approved preparations of A1PI obtained from Cohn fraction IV-1 paste, which differ in the purification methods, concentrations, and dosage forms. Purification methods involve Cohn fractionation, polyethylene glycol (PEG) precipitation or affinity chromatography or by using multiple chromatography steps. Saklatvala and Wood et al. adopted a five step procedure to isolate and purify A1PI which includes precipitation with $(NH4)_2SO_4$, DEAE cellulose and affinity chromatography on concanavalin A sepharose [9]. Most of the inventions in the patents detail the purification methods of A1PI where the starting material is Cohn fraction IV-1 paste and precipitation agents like PEG and $ZnCl_2$ are added to precipitate impurities and A1PI respectively [10,11,12]. The precipitation methods suffer from low yields and less specific activity besides having an additional step included in the process in the form of filtration or centrifugation to separate the precipitate from the supernatant. The methods in which affinity chromatography are used are not suitable for therapeutic protein manufacture due to

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Received May 21, 2015; **Accepted** June 04, 2015; **Published** June 10, 2015

Citation: Kumar NA, Rao KL, Chakraborthy Z, Giri A, Devi KU (2015) Purification of A1PI from Human Plasma-an Improved Process to Achieve Therapeutic Grade Purity. J Chromatogr Sep Tech 6: 277. doi:10.4172/2157-7064.1000277

the strong possibility of ligand leaching. The ligand concanavalin A is listed as being potentially mutagenic and teratogenic [13]. Cohn's ethanol fractionation is the method used by most of the manufacturers. Using ethanol in the process is known to lead to C-terminal lys truncation of A1PI. A linear increase of lys removal with increasing ethanol concentration was reported indicating the usage of ethanol in the process is not desirable to have an intact end product without lysine truncation [14]. The process described herein for human plasma derived A1PI eliminates the need for PEG, $ZnCl_2$, ammonium sulphate precipitation, Cohn's ethanol fractionation or affinity chromatography to purify this protein in high yields. A more efficient process helps to ensure optimal use of a scarce and valuable commodity like human plasma to produce the end product of therapeutic grade.

Materials and Methods

General equipment's in the laboratory

XK16/20 chromatography columns, AKTA Purifier chromatography system, DEAE sepharose resin, capto adhere resin from GE Healthcare, Uppsala, Sweden and Fractogel TMAE resin from EMD-Millipore.

All equipments were from standard manufacturers as given below:

UV Spectrophotometer (GE Healthcare)

pH and Conductivity meter (Thermo Scientific)

Ultra filtration unit-Stirred cell (Amicon, Millipore)

Mini Pellicon TFF system (Millipore)

Electrophoresis unit and power supply (Tarsons)

HPLC system (SHIMADZU-LC2010CHT)

ELISA plate Reader-MULTISKAN EX (Thermo scientific).

Reagents

Substrate: Nα-Benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPA- Sigma/B4875-100 mg)

Trypsin from bovine pancreas (Sigma/T1426-100 mg)

DMSO (Dimethyl sulphoxide) (Sigma/D8418-50 ml)

Primary antibody: Anti human alpha-1-antitrypsin produced in rabbit (A0409-Sigma Aldrich)

Secondary antibody: Anti rabbit IgG ALP conjugated, produced in goat (A3812-Sigma Aldrich)

Coomassie brilliant blue R-250 (11255300251730-Merck)

Chemicals

a) Sodium dihydrogen phosphate monohydrate GR, Di sodium hydrogen phosphate anhydrous GR, Ammonium sulphate GR, Glycine GR, Tris buffer, Sodium acetate trihydrate, Sodium dodecyl sulphate.

A1PI reference standard

α1-Antitrypsin was procured from Sigma-Aldrich. A human plasma-derived protein which is 52 KDa under reducing conditions on SDS-PAGE (Catalog No A9024, Lot No: 018K7546). This was used as a reference standard in this study.

Human plasma

Recovered plasma was procured from licensed and audited blood banks through cold chain shipment. The serology test reports for each of the donor units were verified as negative for HIV 1 and 2 antibodies, HBS Ag, HCV antibody, VDRL and malaria parasite. Plasma bags were also tested negative by nucleic acid testing (NAT for HIV 1 and 2, HBV and HCV). These tested units were taken for the process.

Methodology adopted to purify A1PI

All steps were carried out at room temperature (25°C -27°C). Prior to the purification process, the plasma bags were removed from the freezer (-40°C) and left at room temperature i.e. 25°C for thawing for about 2 hr and then kept for further thawing in water bath set at 25°C -30°C for 30 min. After thawing, the bags were cut open under laminar air flow and the plasma from the bags was pooled in a collection vessel. The pooled plasma was filtered through 40 µm, polypropylene filter (Domnick hunter) followed by a second 15 µm, polypropylene filter (Domnick hunter) to remove the clots or any particulates from plasma. The filtrate was loaded on to chromatography columns to purify A1PI. Filtration was carried out using a peristaltic pump connected to the filters through silicone tubing. Given below was the sequence adopted to purify A1PI to homogeneity (Figure 1).

Capture step of A1PI:

Various chromatography resins were screened to check the efficiency to bind A1PI from human plasma. To capture A1PI from plasma, cation exchange resins like CM sepharose, SP sepharose, eshmunoS; anion exchange resins-DEAE sepharose, eshmuno Q. Hydrophobic interaction chromatography–butyl sepharose, phenyl sepharose and mixed mode resins like capto MMC were tried. Among all the resins, anion exchangers have the ability to bind A1PI and in the group of anion exchangers, particularly DEAE sepharose resin has the highest ability to bind A1PI at pH 5.2 and 2 mS/cm conductivity. So, the column packed with DEAE sepharose resin was optimized as the step to capture A1PI.

The column was packed by flow-packing method at a constant flow rate. Packing buffer was mixed with the resin to form 50-70% slurry. The slurry was poured into the column in a single continuous motion against the column wall to minimize the introduction of air bubbles. After pouring the resin, the adaptor was mounted on the top of the column and connected to a pump. The bottom outlet of the column

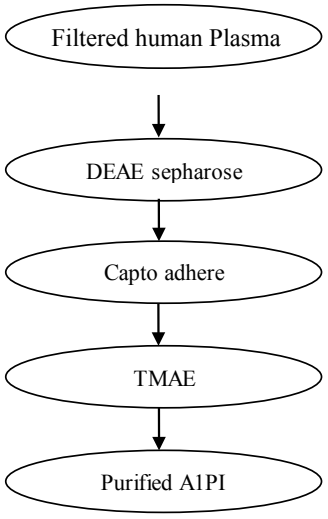


Figure 1: Methodology adopted to purify A1PI.

was opened and the pump was set at flow rate 30% greater than that used during operation. Mobile phase was passed through the column in order to compress the resin. After the bed had compressed, the pump was paused and the adaptor was lowered onto the compressed bed, to complete the packing of the column. The efficiency of the packed column was monitored by determining HETP and asymmetry factor. 1% acetone solution was prepared and applied to the column. The column was washed with water at a flow rate of 30 cm/hr and the washing was continued till the UV peak at 280 nm reached the baseline. HETP and asymmetry values were calculated for this peak to determine the efficiency of the packed column

The filtered plasma without any particulate matter was loaded on to DEAE- sepharose resin packed in XK 16/20 column to a height of 10 cm and this column was equilibrated thoroughly before loading the sample with 50 mM sodium acetate pH 5.2 buffers which is having a conductivity of 2 mS/cm. The column was run at 90 cm/hr-120 cm/hr for loading, washing and elution. The column was washed with the same equilibration buffer to remove the other unbound plasma proteins; 50 mM sodium acetate pH 4.5 buffer was passed through the column to remove other major proteins like albumin from the column. The fraction containing the A1PI protein was eluted with the 50 mM sodium acetate pH 8.0 buffer containing 120 mM NaCl. The column was washed with 0.5M NaCl to remove other tightly bound proteins and 0.5M NaOH was used for regeneration and sanitization.

Other proteins collected in the flow through, wash fraction and regeneration step were not individually characterized in the current research but SDS-PAGE and Western blot analysis indicated that these could be certain major proteins in plasma like albumin, IgG, fibrinogen and others. SDS-PAGE analysis and Western blotting were performed for the column fractions to track A1PI. Western blot analysis confirmed the presence of A1PI in elution peak and SDS-PAGE analysis indicated an A1PI protein band with electrophoretic mobility matching the reference standard

Intermediate purification step of A1PI

A1PI after the initial capture step was loaded on to a second chromatography column for further purification. This step was mainly to separate other co-eluting plasma proteins from A1PI. Q-sepharose, SP sepharose, capto adhere (GEHC), capto MMC phenyl sepharose (GEHC), macro prep methyl (Bio-Rad), cellufine phenyl (JNC) were the different resins tried out for the intermediate purification step of A1PI. Among all the resins, capto adhere which is a strong anion exchanger with multimodal functionality resin was found to give good yield, purity and had better removal capacity for impurities at higher flow rates when compared to other resins which was confirmed by SDS-PAGE analysis. Column was packed with capto adhere resin in XK 16/20 column to a height of 10 cm by flow-packing method as detailed in the above section. 25 mM sodium phosphate buffer containing 20 mM NaCl, pH 7.0 was used for equilibrating the column. DEAE eluate was loaded on to capto adhere column after the conductivity was brought down to 5 mS/cm by diluting with 2 mM sodium phosphate, pH 7.0 buffer. It was observed that A1PI bound to the column and was eluted with 25 mM sodium phosphate buffer containing 150 mM NaCl, pH 7.0. Before elution, the column was washed with 25 mM sodium phosphate buffer containing 70 mM NaCl, pH 7.0 to remove certain impurities whereas the other plasma proteins bound to the column were eluted from the column in 0.5M NaCl wash and regeneration steps. Other conditions tried out were loading conductivity at 2 ms/cm, 10 ms/cm and at pH 8.0 and pH 6.0; elution buffer conductivity of 25 mS/cm, 35 mS/cm but under these conditions A1PI yield and purity was less than the finally chosen method. So, finally loading at pH 7.0, 5 ms/cm and eluting with buffer of pH 7.0 was found to be optimal condition to get purified A1PI in the Elution fraction and to separate it from other plasma proteins. All the fractions from this column were analyzed on SDS-PAGE to check for purity and composition.

Polishing step of A1PI

After the intermediate purification step, A1PI was found to have few more extra bands which were carried over from plasma. In order to purify this further, A1PI protein collected in elution fraction in above step was taken and diluted to 2 mS/cm with 2 mM Tris buffer pH 8.5. The capto adhere column eluate was loaded on to various anion exchange resins to remove the other few impurities and among all the resins, TMAE resin was able to selectively bind A1PI protein. The TMAE resin was packed in XK 16/20 column to a height of 10 cm and equilibrated with 50 mM Tris buffer PH 8.5, conductivity of 2 mS/cm, The above diluted and pH adjusted sample was loaded onto TMAE column at a flow rate of 90 cm/hr. After loading, the column was washed with equilibration buffer to remove any unbound proteins and more impurities were removed from the column by washing the column with 50 mM Tris buffer pH 8.5, containing 75 mM NaCl. Bound A1PI is eluted from the column by changing the pH and ionic strength of the elution buffer and to bring the pH shift, the column was prewashed with 50 mM Tris buffer pH 7.0, conductivity of 2 mS/cm. After the column has attained pH 7.0, elution buffer i.e. 50 mM Tris buffer containing 120 mM NaCl, pH 7.0 was passed through the column to elute A1PI. The impurities which bound to column were observed in the wash fraction and in the cleaning and regeneration peaks. A1PI which was eluted by the buffer of higher ionic strength was found to be free of impurities and homogenous on SDS-PAGE.

Characterization of the purified A1PI

The purified A1PI preparation was analyzed on SDS-PAGE under reducing and non-reducing conditions and on a size exclusion HPLC (SE-HPLC) column. The activity of the purified sample was measured by an enzyme assay method. All the analytical studies were carried out alongside a reference standard (obtained commercially from Sigma).

SDS-PAGE analysis: This was performed to analyze the column fractions and the final purified sample in comparison with a reference standard.

SDS-PAGE methodology: 10% SDS-PAGE gel was made where resolving gel was prepared by adding 3.1 ml of 30% Bis-Acrylamide solution, 1.9 ml of 1.5 M Tris buffer pH 8.8 to 2.5 ml of water. To this solution 37.5 µl of 10% APS and 10 µl TEMED were added and poured into the assembled glass plates and allowed to polymerise. Stacking gel was prepared by adding 0.4 ml of 30% Bis-Acrylamide solution, 0.75 ml of 0.5M Tris buffer pH 6.8 to 1.8 ml of water. To this solution 20 µl of 10% APS and 5 µl TEMED were added and poured on top of the resolving gel. Immediately a clean comb was inserted into the stacking gel carefully avoiding trapping of air bubbles. The gel was allowed to polymerize. After the gel was polymerized the comb was removed carefully. Meanwhile, 40 µl of the samples to be loaded on the gel were aliquoted into micro centrifuge tubes and to that 10 µl of sample buffer (containing Tris, SDS, bromophenol blue, β-mercapto ethanol and glycerol) was added and kept in boiling water bath for 5 min. After boiling, the samples were removed and centrifuged at 10,000 rpm for 2 min. Once the 10% gel was ready, the samples were loaded into the wells slowly using micropipette. The casting unit was filled with tank buffer (containing Tris, glycine and SDS) and electrophoresis was carried out at 300 V for 45 min till the tracking dye (bromophenol

blue) had run out of the gel. Then the gel was carefully removed and stained with Coomassie brilliant blue R-250 dye prepared in methanol and acetic acid. Once the protein bands were developed, the gel was destained using solution containing methanol and acetic acid. Then the gel was scanned and documented.

Western blot methodology: The protein bands were separated on 10% SDS-PAGE as detailed above and the gel was trans-blotted onto a nitrocellulose (NC) membrane. Transfer was carried out for 2 hrs at 100 V in a buffer containing Tris–HCl, glycine and methanol. After transfer, the NC membrane was incubated in 50 ml TBST (Tris buffered saline with Tween-20) containing 5% skimmed milk powder for 60 min. This step was to block additional protein binding sites. Following the incubation, the NC membrane was washed with TBST twice for 10-15 min. After washing, the membrane was incubated with 25 mL anti human alpha-1-antitrypsin antibody produced in rabbit (Polyclonal IgG, 0.5 mg/mL stock diluted 1:10,000 in TBST buffer containing 0.25% Bovine serum albumin) for 1 hr at room temperature with gentle shaking. The membrane was washed thrice for 10 min in 50 ml TBST and then incubated the membrane in 25 mL Anti rabbit IgG ALP Conjugated antibody (1:30,000 dilution with TBST) at room temperature for 1 hr with gentle shaking. After 1 hr, the blot was washed with TBST and developed with 3,3',5,5'N-tetramethylbenzene substrate which is a chromogenic substrate added to develop the specific protein bands which reacted with the antibodies.

SE-HPLC: SE-HPLC method was used to characterize the purified molecule. The analysis was carried on a Shodex (vendor) Protein KW-803 Shodex column using sodium phosphate pH 7.0 buffer.

Specific activity: The alpha1-antitrypsin (AAT) activity of the purified A1PI was measured using an enzyme assay method which is based on the inhibition of trypsin activity by AAT and estimation of the residual trypsin activity using a synthetic substrate benzoyl-DL-arginine-P-nitroanilide (BAPA). The level of the product (p-Nitroaniline) formed was estimated by measuring the absorbance at 410 nm. The absorbance was read at 410 nm after the reaction was terminated using MULTISKAN EX reader (Thermo scientific) 0.15 ml of trypsin was mixed with 0.006 ml of test sample or standard and 0.144 ml of 50 mM Tris buffer, pH 8.2 with 20mM $CaCl_2$ and this mixture was incubated for 5 minutes at 37°C. Then 3.5 ml of benzoyl-DL-arginine-P-nitroanilide (BAPA) was added to the above solution and incubated for 10mins at 37°C. The reaction was stopped by addition of 0.5 ml of 30% acetic acid and the OD was measured at 410 nm against a suitable blank [15,16].

Excess Trypsin+ AAT →Trypsin-AAT complex+ Residual Trypsin

Residual Trypsin+BAPA→Nα-Benzoyl-DL-arginine+P-Nitro aniline

Results and Discussion

DEAE sepharose step to capture A1PI

DEAE-sepharose resin was packed in XK 16/20 column to a height of 10 cm and this column was equilibrated with 50 mM sodium acetate pH 5.2 buffers with a conductivity of 2 mS/cm. The column was run at 90 cm/hr-120 cm/hr for loading, washing and elution. The column was washed with the same equilibration buffer to remove the other unbound plasma proteins and then passed wash-2 buffer i.e. 50 mM sodium acetate pH 4.5 buffer to remove other bound proteins like albumin and clotting factors. A1PI which was bound to the column was eluted with the 50 mM sodium acetate pH 8.0 buffer containing 120 mM NaCl, conductivity of 18 mS/cm (DEAE Chromatogram Figure 2a). The column was washed with buffer containing 0.5M NaCl to remove the tightly bound proteins and 0.5M NaOH for regeneration and sanitization. SDS-PAGE analysis and western blotting was performed for the column fractions to track for A1PI (Figures 2b and 2c). Western blot analysis indicated the presence of A1PI band in the eluate fraction and its mobility is matching to the reference standard.

Capto adhere step

The DEAE- sepharose eluate with other impurities was loaded on to capto adhere multi modal strong anion exchange column after the conductivity and pH were adjusted to 5 ms/cm and 7.0 respectively. The column was equilibrated with 25 mM sodium phosphate buffer containing 20 mM NaCl, pH 7.0 and Conductivity of 5 mS/cm. The sample was loaded on to the equilibrated column at 90 cm/hr flow rate and under the applied conditions A1PI bound to the column and some impurities observed in Flow through fraction. The column was washed with wash-2 buffer of higher conductivity to remove other proteins (Figure 3a). A1PI bound to the column was eluted with buffer at 20 mS/cm conductivity. Eluate sample was analyzed on SDS-PAGE. The A1PI band was enriched in intensity but it was also found to have a prominent low molecular weight impurity and few high molecular weight impurities (Figure 3b). In order to remove these impurities, the eluate sample is further loaded on to TMAE column.

TMAE step to purify A1PI to homogeneity

The TMAE resin was packed in XK 16/20 column to a height of 10 cm and was equilibrated with 50 mM Tris pH 8.5 buffers which are having a conductivity of 2 mS/cm. The eluate from capto adhere column after adjusting its conductivity to 2 mS/cm was loaded onto TMAE column at a flow rate of 90 cm/hr and after loading the column was washed with equilibration buffer and wash-2 buffer to remove any unbound proteins and to separate the other bounded impurities from the A1PI. After passing wash-2 buffer, pre elution buffer was passed through the column to shift the pH of column to 7.0. A1PI bound to the column was eluted by changing the pH and ionic strength of the buffer and elution was carried out with 50 mM Tris pH 7.0 buffer containing 120 mM NaCl, conductivity of 14 mS/cm. The strongly bound impurities were removed by washing the column with 0.5M NaCl and 0.5M NaOH solutions (Figure 4a). All the fractions from the column were analyzed on SDS-PAGE and the analysis indicated that the low molecular weight impurity bound strongly to the column under the applied conditions and was observed in 0.5M NaCl wash. The sample from the elution peak showed a single band on SDS-PAGE (Figure 4b). The eluate from TMAE column is concentrated and subsequently exchanged into 20 mM sodium phosphate buffer pH 6.8, containing 0.1M sodium chloride. This buffer exchange is carried out using 10

Dilution Folds	Content of total protein in reaction(mg)-test sample	OD at 410 nm	Active A1PI (mg)	Active A1PI per mg of total A1PI	% of active A1PI
Neat	0.0047	0.902	0.0045	0.957	95.7

Table 1a: The data indicates that 0.957 mg of active A1PI is present in 1 mg of total protein.

Dilution folds	Content of Total Protein in Reaction(mg)-Test sample	Active A1PI (mg)	Content of Trypsin Inhibited (mg)	A1PI inhibiting 1mg of trypsin (mg)
Neat	0.0047	0.0045	0.0026	1.8

Table 1b: The data indicates that 0.957 mg of active A1PI is present in 1 mg of total protein.

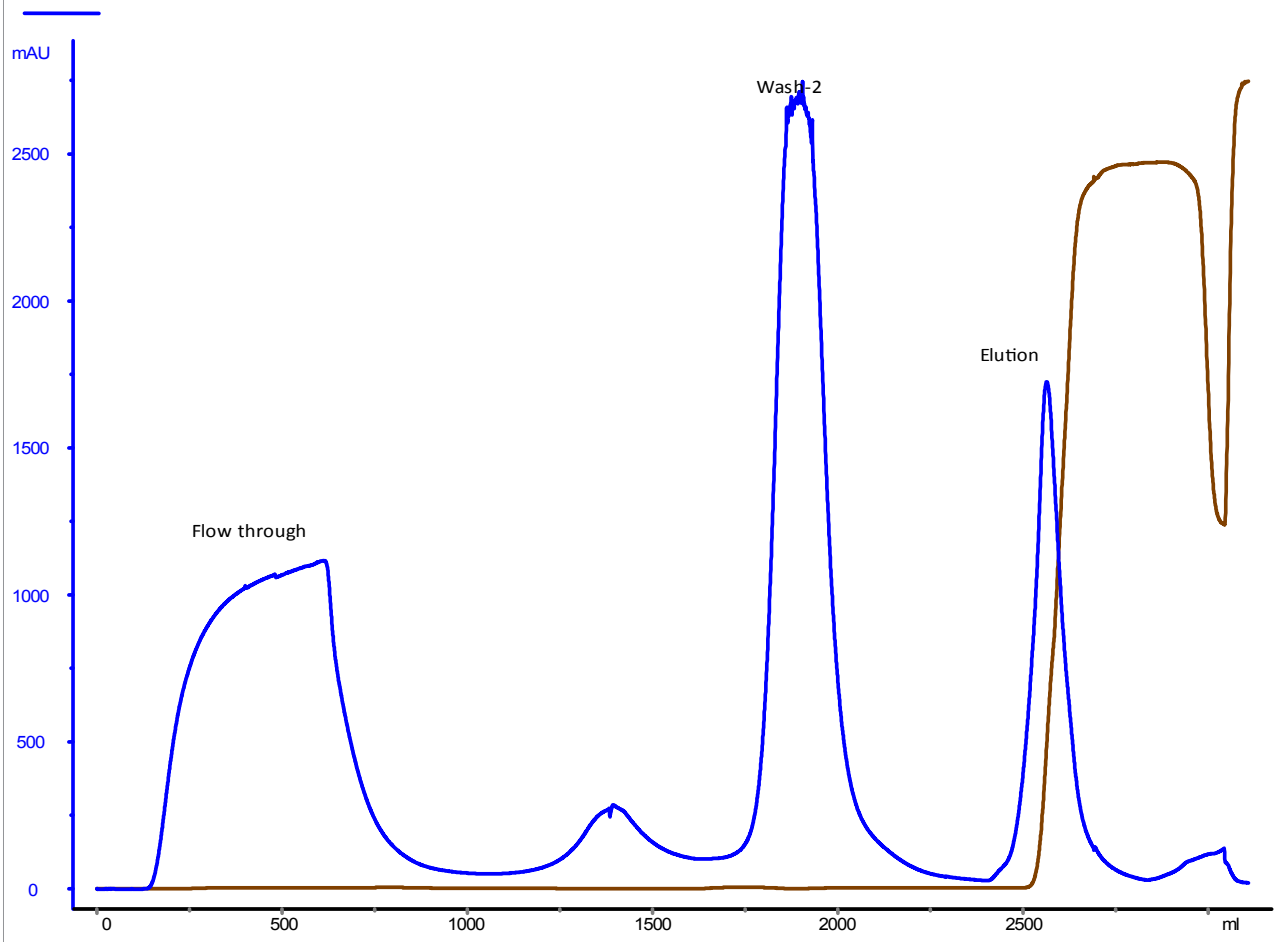


Figure 2a: DEAE Sepharose FF chromatography (1 × 10 cm, 20 ml) Equilibration buffer: 50 mM sodium acetate pH 5.2, Wash-1: Equilibration buffer, wash-2 buffer: 50 mM sodium acetate pH 4.5, elution buffer: 50 mM sodium acetate pH 8.0 containing 120 mM NaCl, Cleaning buffer: 50 mM sodium acetate pH 8.0 containing 0.5M NaCl.

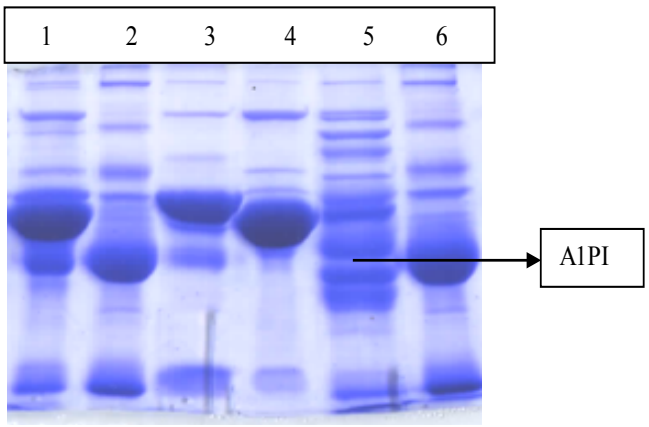


Figure 2b: SDS-PAGE analysis was performed to analyze the DEAE fractions. Samples were prepared in loading buffer containing 60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue and run on a 10% polyacrylamide gel. Lane 1: Load sample, + βME; Lane 2: Flow through, +βME; Lane 3: Wash-1, +βME; Lane 4: Wash-2, +βME; Lane 5: Elution peak sample, +βME; Lane 6: Cleaning peak sample, +βME.

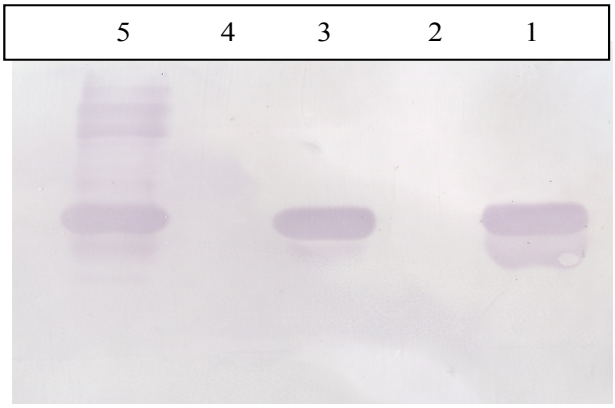


Figure 2c: Western blot analysis was performed to identify the fraction containing A1PI protein. Samples were prepared in loading buffer containing 60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue and run on a 10% polyacrylamide gel and the gel was transferred on to a blot. Lane 1: Load sample; Lane 2: Wash-2 sample; Lane 3: Elution peak sample; Lane 4: Cleaning peak sample; Lane 5: Sigma Aldrich Standard.

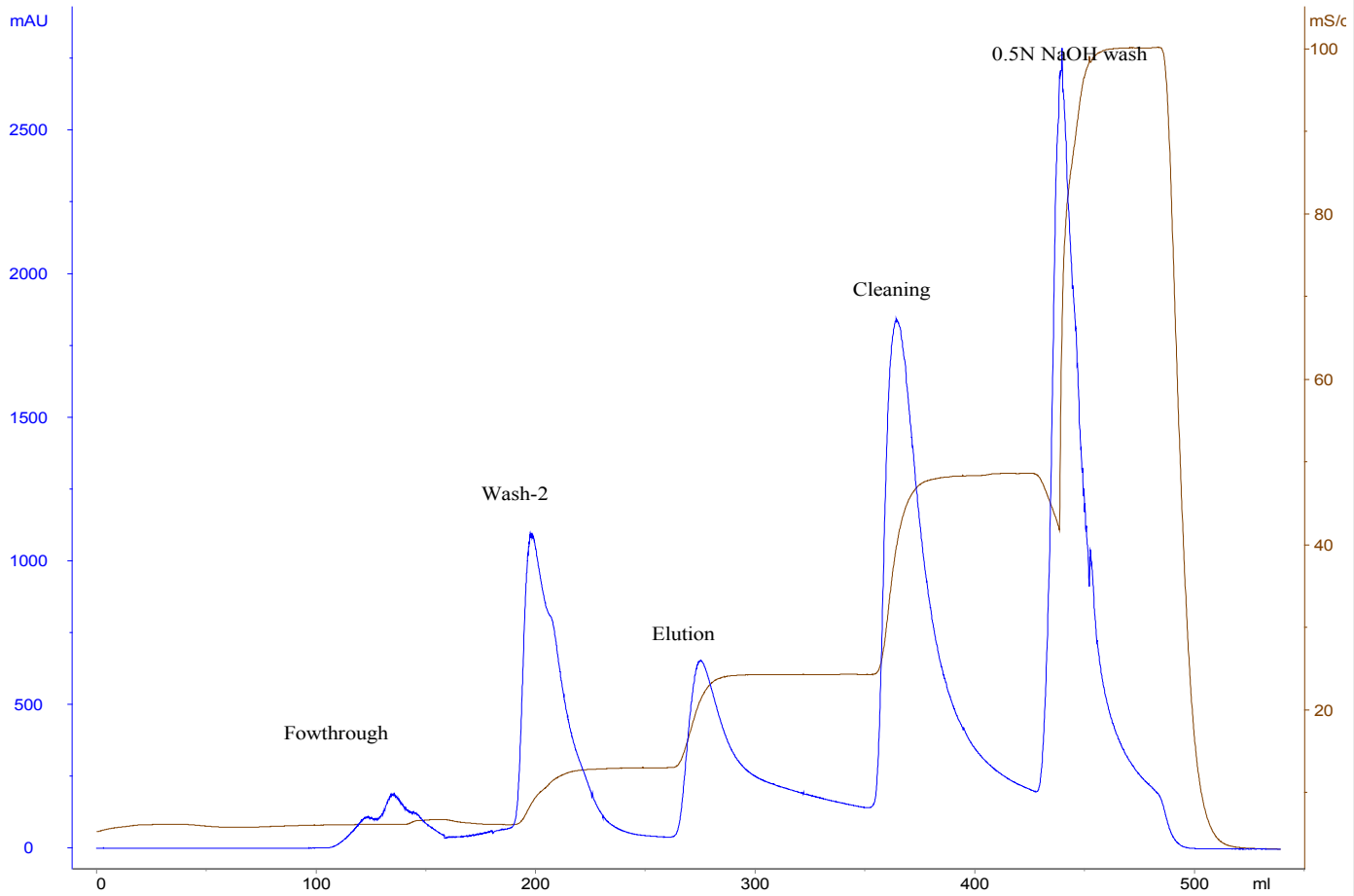


Figure 3a: Capto adhere multi modal chromatography for the DEAE elution fraction on a 20 ml column. A1PI bound to the column and was observed in the elution fraction.

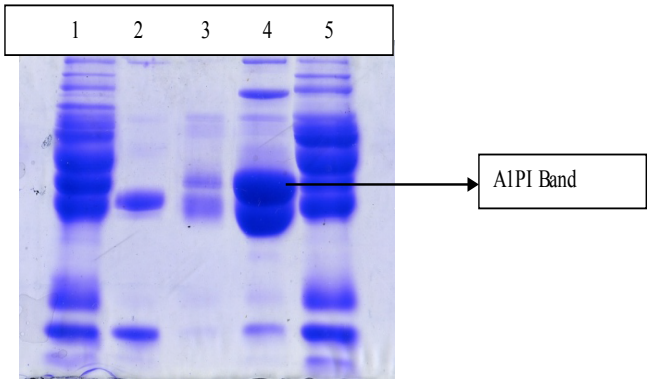


Figure 3b: SDS-PAGE analysis was performed to analyze the capto adhere column fractions. Samples were prepared in loading buffer containing 60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue and run on a 10% polyacrylamide gel. Lane 1: Load sample, + βME; Lane 2: Flow through, +βME; Lane 3: Wash-2, +βME; Lane 4: Lane 4: Elution peak sample, +βME; Lane 5: Cleaning peak sample, +βME.

KDa TFF cassettes on a mini Pellicon TFF system. The observed yield is 0.6-0.7 g of A1PI per liter of human plasma by adapting the developed process and the purified molecule is characterized by the methods described in the following section.

SDS-PAGE analysis of the purified A1PI

The eluate after TMAE step is analyzed on SDS- polyacrylamide gel electrophoresis (Figure 5) and is found be more than 95% pure. On comparison with reference standard its molecular weight is around 52 kDa under both reducing conditions and non-reducing conditions and the mobility matched that of the reference standard.

SE-HPLC analysis of the purified A1PI

The identity and purity check by SE-HPLC analysis in comparison to standard indicated that the purified A1PI protein is close to 93% pure (Figure 6a and 6b). The analysis was carried out on Protein KW-803 Shodex column (8.0 mm I.D × 300 mm length) with Particle size of 5 µm. The retention time of the purified A1PI peak (9.313 min) closely matched the retention time of the reference standard (9.293 min) and also showed a better purity profile (93%) with a major single peak and a minor peak at 7.771 min, when compared to the reference standard (75% purity) with at least three other contaminants on SE-HPLC chromatogram. Sodium phosphate buffer containing NaCl was the buffer used for running the column at 1 ml/min flow rate. A pilot study was carried out by ZLB Behring LLC to compare the purity, functionality and isoform composition of the available market products (Aralast, Prolastin and Zemaira). SEC-HPLC comparison indicated that Zemaira was 95.98%, Prolastin 79.00% and Aralast 63.55% monomeric [17]. In-house purified A1PI was found to have 93% monomeric form.

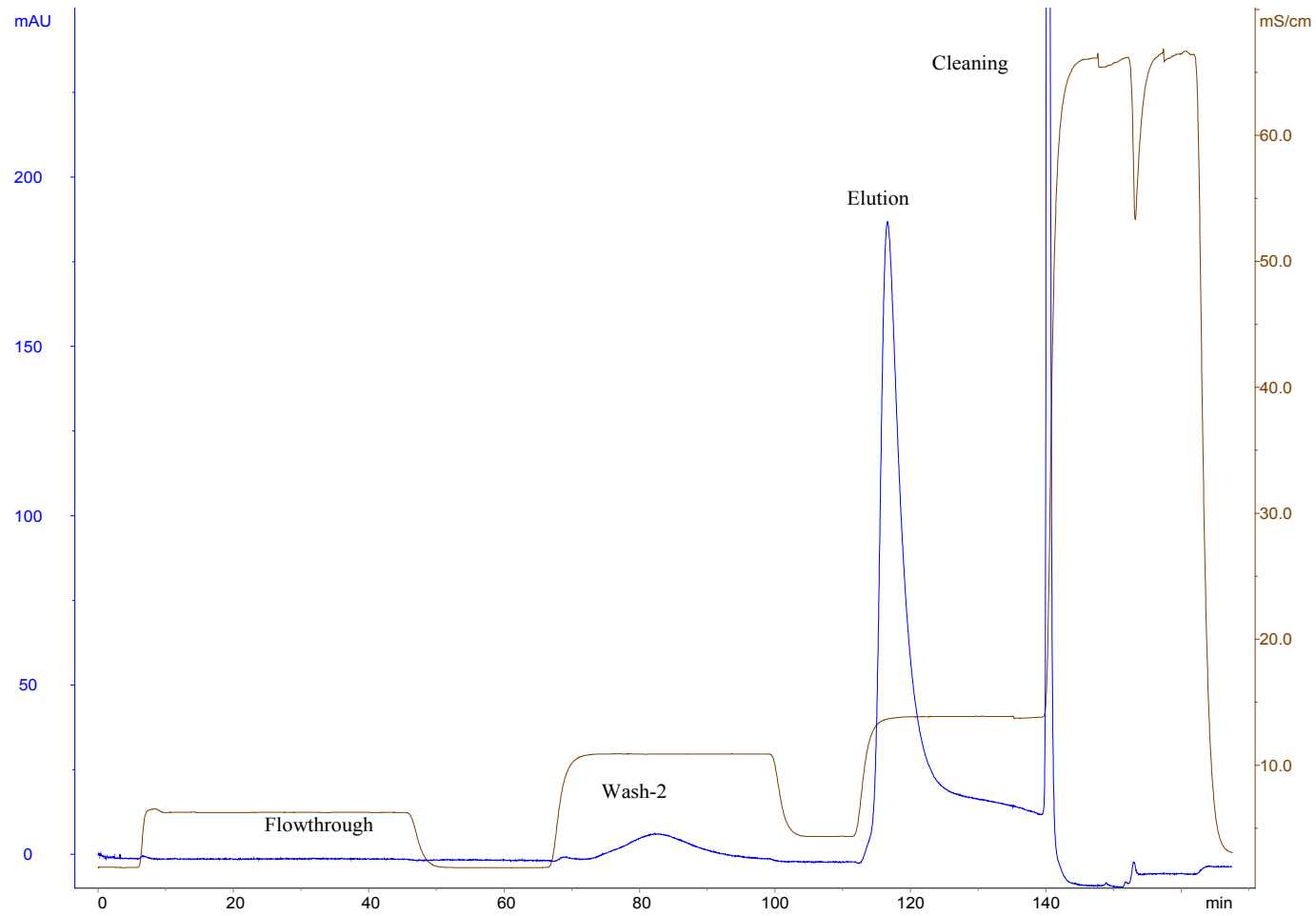


Figure 4a: TMAE chromatography for the capto adhere eluate on a (1 × 10 cm, 20 ml) column. Equilibration: 50 mM Tris buffer PH 8.5, conductivity 2 mS/cm. Wash-2: 50 mM Tris buffer PH 8.5, containing 75 mM NaCl.10 mS/cm. Wash-3: 50 mM Tris buffer PH 7.0, conductivity 2 mS/cm. Elution: 50 mM Tris buffer containing 120 mM NaCl, pH 7.0 and conductivity 14 mS/cm.

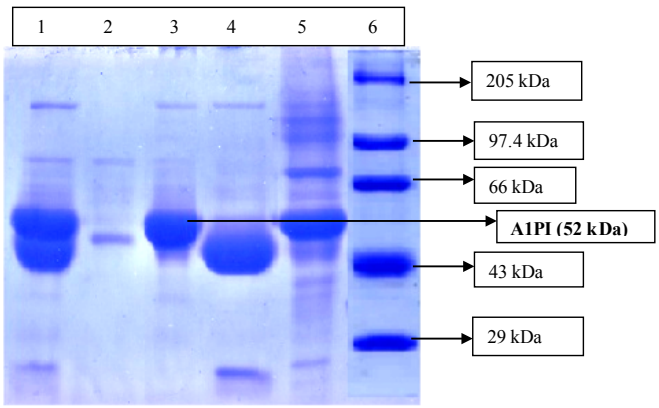


Figure 4b: SDS-PAGE analysis was performed to analyze the fractions from TMAE column. Samples were prepared in loading buffer containing 60 mM Tris -Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue and run on a 10% polyacrylamide gel. Lane 1: Load sample, +βME; Lane 2: Wash-2 Peak, +βME; Lane 3: Elution peak sample; Lane 4: 0.5M NaCl wash peak, +βME; Lane 5: 0.5N NaOH wash peak, +βME; Lane 6: Molecular weight marker, 29-205 KDa.

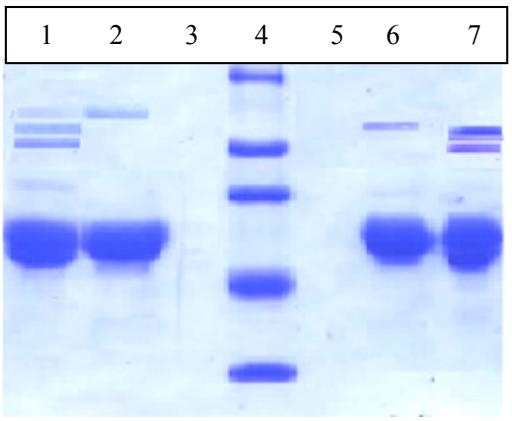
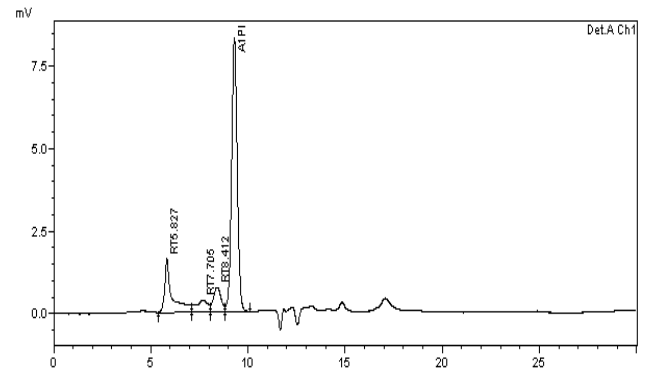


Figure 5: SDS-PAGE analysis was performed to check the purity of A1PI protein in reference to standard (Sigma). Samples were prepared in loading buffer containing 60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue and run on a 10% polyacrylamide gel. Lane 1: Reference standard 15 µg, -βME; Lane 2: Purified sample 15 µg, -βME; Lane 3: Empty well; Lane 4: Molecular weight marker, 29-205 KDa; Lane 5: Empty well; Lane 6: Purified sample 15 µg, +βME; Lane 7: Reference standard 15 µg, +βME.

(a) Reference standard for A1PI (Sigma Aldrich).



(b) In-house purified A1PI molecule.

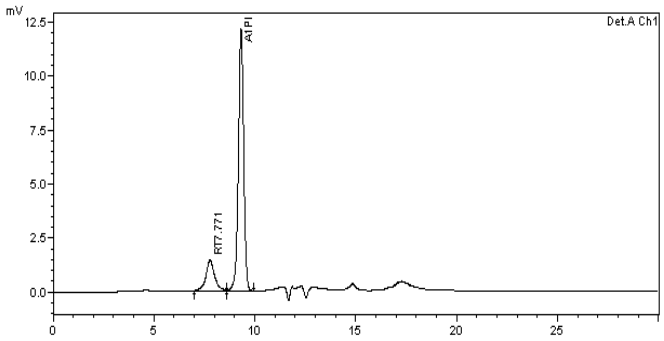


Figure 6: SE-HPLC analysis was performed to check the purity of purified A1PI protein in reference to standard shows that (a) Reference standard is 75% pure. (b) In-house purified A1PI molecule is 93% pure.

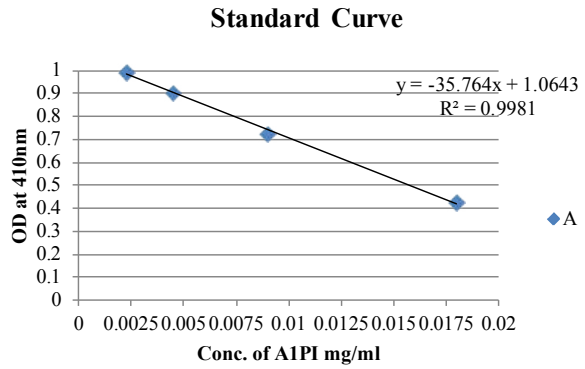


Figure 7: O.D. at 410 nm was plotted versus concentration of A1PI (mg/ml) using MS-Excel software to prepare a standard A1PI activity curve.

Dilution folds	Content of ref. std. in reaction mixture (mg)	Content of trypsin inhibited (mg)	OD at 410 nm
1.7	0.018	0.0105	0.427
3.4	0.009	0.0053	0.727
6.8	0.0045	0.0026	0.904
13.6	0.00225	0.0013	0.992

Table 2: A standard curve was plotted at different concentrations of A1PI as given below:

Specific activity

The specific activity (mg of A1PI/mg of protein) of purified A1PI is determined with reference to a standard A1PI activity curve. This assay was carried out along with the reference standard from Sigma and considered the below information for the assay - alpha-1-antitrypsin from human plasma (Sigma) was used and as per the label claim 1.7 mg of alpha 1 proteinase inhibitor inhibits 1.0 mg of trypsin (Figure 7 and Table 2). The purified and concentrated TMAE eluate was tested in this assay for its ability to inhibit trypsin and to calculate the percent of active protein in the purified fraction (Table 1a and 1b). Pilot study carried out by ZLB Behring LLC to compare the specific activity of the available market products indicated the specific activity (activity/mg protein) values were 0.85, 0.65 and 0.56 for Zemaira, Aralast and Prolastin respectively [17]. So, the in-house purified A1PI has specific activity comparable to Zemaira.

Conclusion

Since 1987, several A1PI products derived from pooled human plasma have been approved and are currently available to slow down the progression of emphysematous conditions in A1PI deficient patients. In addition, due to its multiple physiological activities, A1PI has been identified for its involvement in several other rare diseases, the treatment of which may possibly benefit from A1PI based therapies. From this standpoint, the product quality of the purified preparations for therapeutic use is very important [8]. There are many reports of purification of A1PI by using precipitation methods with PEG and $ZnCl_2$ [18-22]. In the above experiments, an attempt was made to look for a purification scheme that is both economical and scalable for the purification of A1PI, without compromising on yields, purity and its other therapeutic characteristics. After an initial capture on DEAE resin, two different new generation resins were chosen for their ability to selectively bind and purify A1PI from among the other plasma proteins. Figures 3a and 3b indicate how the use of capto adhere resin was able to purify A1PI to a significant extent with the SDS-PAGE (Figure 3b) analysis revealing only a few protein impurities. As a polishing step for purity, the A1PI sample after Capto adhere step was loaded on Fractogel TMAE resin, which was successful in increasing the purity to a significant extent as shown in lane 3 of Figure 4b. The yield of the final purified A1PI by this process was found to be in the range 0.6-0.7 g/litre of human plasma. This increased recovery and purity helped us nail this as an ideal and improved process scheme for therapeutic manufacturing, subject to the other biochemical characteristics being within the required specifications. Results of the characterization studies on purified A1PI indicated the comparability of the protein with an existing reference standard with respect to its purity on SDS-PAGE, SE-HPLC analysis and specific activity (Figures 4-7). The specific activity results show that the functionality of the protein has not been compromised by this purification scheme. A scheme that uses only chromatography steps is easily scalable unlike the earlier reported procedures of precipitation and affinity capture. The increased yields and purity show that further studies can be done to adapt the method for industrial manufacturing of this very important therapeutic protein from human plasma.

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