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C1-Esterase Inhibitor from Human Plasma-An Improved Process to Achieve Therapeutic Grade Purity

Nuvula Ashok Kumar*, Korla Lakshmana Rao, Archana Giri and Komath Uma Devi

Centre for Biotechnology, JNTUH, Hyderabad, India

Abstract

C1-Esterase Inhibitor (C1-INH) is a protein present in human plasma and is a member of serine protease inhibitors super family. C1-INH is a molecule with lot of therapeutic importance and is widely used in the treatment of Angioedema and in the current research an improved purification scheme is developed to fractionate C1-INH from human plasma. By a series of chromatography steps C1-INH is purified to homogeneity. A simple three step chromatography procedure has been described excluding the more inefficient or cumbersome methods like precipitation or affinity capture. The C1-INH purified by this scheme is characterized along with the reference molecule. C1-INH produced by this method is a single-chain glycoprotein with a molecular weight of 85-93 KDa with increased purity and yields of 100-120 mg/L of plasma. The chromatography steps are easily scalable and the process is economical and adaptable for therapeutic protein manufacture.

Keywords: C1-esterase inhibitor; Cellufine phenyl; DEAE Sepharose; Fractogel TMAE; IC_{50}

Abbreviations: C1-INH: C1 Esterase Inhibitor; PEG: Poly Ethylene Glycol; DEAE: Diethyl Amino Ethyl; TMAE: Tri Methyl Amino Ethyl; IC_{50}: Half Maximal Inhibitory Concentration

Introduction

Deficiency of C1-INH results in Hereditary Angioedema (HAE) which is characterized by localized swelling in the extremities, face, gut or upper airways; the most serious and potentially life-threatening manifestation of the disease is laryngeal edema [1,2]. Based on the combination of total protein and functional C1- INH levels, there are 3 types of HAE. Type-I is because of deficiency in the amount of C1-INH protein present in the plasma. In type-II, the circulating C1-INH concentration is normal or high but not fully functional where as type-III is acquired deficiency which is very rare. In type III HAE, there is no abnormality found with C1 inhibitor. Instead, it has several potential causes. This condition occurs mainly in women and can be made worse with pregnancy or use of birth control medications. It is also associated with a mutation in the Factor XII gene (F12 gene). In people with type III HAE, too much coagulation Factor XII is produced. C1-INH is administered to treat HAE but studies in animals and observations in patients indicated that administration of C1-INH may have a beneficial effect as well in other clinical conditions such as sepsis, cytokine-induced vascular leak syndrome, acute myocardial infarction, or other diseases [2]. C1-INH inhibits factor XIIa, factor XII fragment (XIIf), kallikrein, and plasmin. Thus, in its absence, there is marked activation of the bradykinin-forming cascade which is a vasodilator resulting in severe angioedema. [2].

C1-INH is a protein derived from human plasma. It is a member of serine proteinase inhibitor (serpin) super family. Other proteinases in this family are: Alpha-1-antitrypsin, Antithrombin, Alpha-2-macroglobulin, Heparin cofactor II, and Alpha-2-antiplasmin [1,2]. C1-INH is a major regulator of the classical complement pathway, contact activation pathway and fibrinolytic pathway and represents the only inhibitor of the classical pathway proteases-C1r and C1s [1-3]. C1-INH is a single polypeptide chain containing 478 amino acid residues with a molecular weight of about 90-105 KDa under non-reducing and 85-93 KDa under reducing conditions derived from human plasma. C1-INH protein isoelectric point lies near 2.7-2.8 [1]. The high degree of glycosylation is unique to C1-INH among human blood plasma proteins, which is heavily glycosylated with approximately 50% of its total weight is contributed by carbohydrates; the molecular weight of peptide chain is ~53 KDa [1,3]. During the initial investigations that resulted in the isolation and characterization of C1, human serum was found to contain a heat sensitive substance that inhibited the enzymatic activity of C1. This inhibitory activity, termed CI esterase inhibitor, was further defined by Lepow [4] and was first isolated by Pensky et al. [5].

Several methods have been described for the purification of C1-esterase inhibitor involving Polyethylene Glycol (PEG) precipitation [6,7] or affinity chromatography or by using multiple chromatography steps [8-11]. The precipitation method suffers from low yields and less specific activity and the affinity method with lectin columns are not suitable for therapeutic protein manufacture due to the strong possibility of ligand leaching. The process described herein for human plasma derived C1-INH eliminates the need for PEG precipitation 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. It also ensures a more desirable quality for the end product by continuously upgrading the existing technology.

Materials and Methods

General equipment's in the laboratory

XK16/20 chromatography columns, AKTA Purifier chromatography system, DEAE Sepharose resin from GE Healthcare,

***Corresponding author:** Ashok Kumar N, Centre for Biotechnology, JNTUH, Hyderabad, Telangana, India, Tel: 91-986-669-6098; E-mail: ashnuvvula@gmail.com

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Uppsala, Sweden. Cellufine phenyl resin from JNC Corporation and Fractogel TMAE resin from EMD-Millipore.

All equipments were from standard manufacturers as given below.

UV Spectrophotometer (GE Health care)

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: Z-Lys-SBzl (N-Carbo benzyloxy-Lys-thiobenzyl ester) (Sigma), Primary antibody: Mouse monoclonal C1-Inhibitor IgG1 antibody (R&D systems), Secondary antibody: Rabbit polyclonal against mouse IgG1 (Sigma Aldrich), Coomassie brilliant blue R-250 (Merck), DMSO (Dimethyl sulphoxide) (Sigma), DTNB (5,5'-Dithio bis (2 nitro benzoic acid) (Calbiochem).

Chemicals

Citric acid anhydrous GR, Tri Sodium Citrate dihydrate GR, Sodium Dihydrogen phosphate monohydrate GR, Di Sodium hydrogen phosphate anhydrous GR, Ammonium Sulphate GR, Glycine GR, Tris buffer, Sodium acetate trihydrate, Sodium dodecyl sulphate. All reagents and chemicals used were from Merck.

C1-INH Reference standard

This was procured from R&D systems. A human plasma-derived protein which is 93 KDa under reducing conditions on SDS-PAGE (Catalog No 2488-PI-200, Lot No: NNH0111111, Make: R&D systems). In this study this was used as a reference standard. Purity of reference standard is >95% by SDS-PAGE under reducing conditions. Storage temperature is -20°C to -70°C for the lyophilized form which is stable for 6 months and after reconstitution, it is stable for 3 months at -20°C to -70°C.

Recombinant C1s

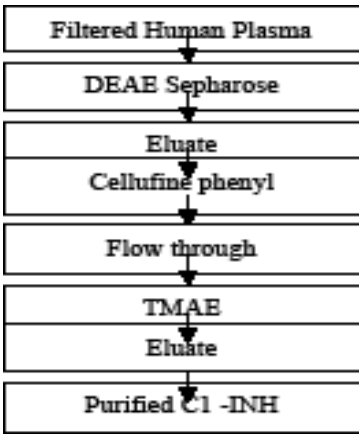
This was procured from R&D systems. It was a homogeneous protein on SDS-polyacrylamide gel electrophoresis which could form a stable complex with C1 esterase inhibitor, if this was added in excess (Catalog No: 2060-SE-010, Lot No: MTX0512051, Make: R&D systems). Purity of recombinant C1S is >95% by SDS-PAGE under reducing conditions. Storage temperature is -20°C to -70°C for the lyophilized form which is stable for 6 months and after reconstitution, it is stable for 3 months at -20°C to -70°C.

Human plasma for developing the process

Recovered plasma was procured from licensed and audited blood banks through cold chain shipment. Plasma was collected from the blood banks in and around Hyderabad, Visakhapatnam, and Bangalore which are part of the southern region of India. 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 adapted to purify C1-INH

All steps were carried out at room temperature (25-27°C). Prior to the purification process, the 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 15 µm, Polypropylene filter (Domnick hunter) to remove the clots or any particulates and the filtrate was loaded on to chromatography columns to purify C1-INH. Filtration was carried out using a peristaltic pump. Below given was the sequence adapted to purify C1-INH to homogeneity.



Capture step of C1-INH

Various chromatography resins were screened to check the efficiency to bind C1-INH from human plasma. To capture C1-INH from plasma, Cation exchange resins like CM Sepharose, SP Sepharose, Eshmuno S; Anion exchange resins-DEAE Sepharose,

Q Sepharose, Eshmuno Q and Mixed mode resin like Capto MMC were tried. Among all the resins, anion exchangers have the ability to bind C1-INH and in the group of anion exchangers, particularly DEAE Sepharose resin has the highest ability to bind C1-INH at pH 7.0 and 5 mS/cm conductivity. So, the column packed with DEAE Sepharose resin was optimized as the step to capture C1-INH.

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 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 20 mM Sodium citrate pH 7.0 buffers which is having a conductivity of 5 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. The fraction containing the C1-INH protein was eluted with the same buffer having increased conductivity of 20 mS/cm. The column was washed with 0.5 M NaCl to remove other tightly bound proteins and 0.5 M NaOH 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 were certain major proteins in plasma like Albumin, IgG, clotting factors etc. SDS-PAGE analysis and western blotting was performed for the column fractions to track for C1-INH .Western blot analysis confirmed the presence of C1-INH in elution-1 peak and SDS-PAGE picture indicated C1-INH protein band with less intensity and its electrophoretic mobility was matching to the reference standard.

Intermediate purification step of C1-INH

C1-INH after the initial capture step with lot of other plasma proteins was loaded on to the second chromatography column for purification. This step was mainly aimed to separate the other plasma proteins from C1-INH. Butyl Sepharose (GEHC), Phenyl Sepharose (GEHC), Octyl Sepharose (GEHC), Macro prep methyl (Bio-Rad), Cellufine phenyl (JNC) were the different hydrophobic resins tried out for the intermediate purification step of C1-INH but among all the resins, Cellufine phenyl 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 Cellufine phenyl 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 0.8 M $(NH_4)_2SO_4$ was used for equilibrating the column and the same concentration of $(NH_4)_2SO_4$ was used for preparing the load and at this concentration of $(NH_4)_2SO_4$, it was observed that C1-INH did not bind to the column and was collected in the flow through fraction whereas the other plasma proteins bound to the column and eluted in the column water wash and regeneration steps. Other concentrations tried out were 0.4 M and 0.6 M $(NH_4)_2SO_4$ but under these conditions, C1-INH has bound to the column along with other proteins and at 1.0 M and 1.2 M $(NH_4)_2SO_4$ concentrations, precipitation was observed in the load sample and the sample became hazy. So, finally 0.8 M $(NH_4)_2SO_4$ concentration was found to be optimal to get purified C1-INH in the flow through fraction and to separate it from other plasma proteins. All the fractions from this column were analyzed on SDS-PAGE to check for the purity.

Polishing step of C1-INH

After the intermediate purification step, C1-INH was found to have two more extra bands which are carried over from plasma but not degradants of C1-INH which was confirmed by carrying out Western blot analysis for the purified C1-INH where only C1-INH band reacted with the antibodies and the two extra bands were not picked up in Western blot. Hence, those extra bands were presumed to belong to other proteins in plasma with lower molecular weights than C1-INH. In order to purify this further to homogeneity, C1-INH protein collected in flow through fraction in above step was diafiltered into 25 mM sodium citrate pH 7.0 buffer to remove ammonium sulfate. This diafiltered sample was loaded on to various cation exchanger resins like CM Sepharose, SP Sepharose, Eshmuno S and TMAE to remove the two extra bands and among all the resins, TMAE resin was able to selectively bind C1-INH protein. The TMAE resin was packed in XK 16/20 column to a height of 10 cm and equilibrated with 25 mM sodium citrate buffer containing 30 mM NaCl which is having a pH of 6.0 and conductivity of 8 mS/cm. The diafiltered sample was loaded onto TMAE column at a flow rate of 90 cm/hr and after loading the column was washed with equilibration buffer to remove any unbound proteins. C1-INH bound to the column was eluted by changing the pH and ionic strength of the buffer. Elution was carried out with 25 mM Sodium citrate containing 170 mM NaCl which is having a pH of 7.0 and Conductivity of 20 mS/cm. The impurities which did not bind to column were observed in the flow through fraction. The bound C1-INH which was eluted by the buffer of higher ionic strength was found to be free of impurities and was looking pure on SDS-PAGE.

Characterization of the purified c1-esterase inhibitor

The purified C1-INH preparation was analyzed for homogeneity on SDS-PAGE under reducing and non-reducing conditions, on Size Exclusion HPLC (SE-HPLC) and Reverse phase–HPLC (RP-HPLC) columns. All the analytical studies were carried out alongside a reference standard (obtained commercially from R&D systems).

a) SDS-PAGE analysis was performed to analyze the fractions and also the purified sample in comparison with the reference standard.

SDS-PAGE methodology

10% SDS-PAGE gel was casted 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 gel plates and allowed to polymerise. Stacking gel was prepared by adding 0.4 ml of 30% Bis-Acrylamide solution, 0.75 ml of 0.5 M 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 has 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 is ready, the samples were loaded into the wells slowly using micropipette. Then filled the gel and the casting unit with tank buffer (containing Tris, Glycine and SDS) and carried out electrophoresis at 300 V for 45 min till the tracking dye (bromophenol blue) has 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 this SDS-PAGE 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 serpin G1/C1 inhibitor Antibody (monoclonal mouse IgG, 0.5 mg/mL stock diluted 1:500 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 Rabbit anti-mouse-IgG polyclonal antibody conjugated to horseradish Peroxidase (1:1000 dilution with TBST) at room temperature for 1 hr with gentle shaking. After 1hr, the blot was washed with TBST. 1X TMB-H_2O_2 which is a chromogenic substrate was added to develop the specific protein bands which reacted with the antibodies.

b) SE-HPLC and RP-HPLC methods were developed to characterize the purified molecule. RP-HPLC analysis was carried out in gradient mode on Inertsil c4 column using Acetonitrile/TFA solvent system. SE-HPLC analysis was carried on Shodex Protein KW-803 shodex column using Sodium citrate pH 6.8 buffers.

c) The specific activity of the purified C1-INH protein was estimated in comparison with the reference standard by measuring the IC_{50} using a colorimetric method. The method [12,13] is based on the ability of C1-INH to inhibit the recombinant human complement C1s and estimation of the residual C1s esterase activity by measuring the increase in absorbance at 405 nm in a kinetic test using colorimetric peptide substrate Z-Lys-SBZl. The OD values obtained for C1-INH reference standard and in-house purified sample are plotted on Graph Pad prism. The activity of C1-INH may also be measured by checking its ability to prevent the release of H^+ ions which is monitored by the use of pH meter or a suitable acid base indicator [14]. The commercially available methods depends on measuring the C1 INH-C1S complex formation which is an immune assay method or inhibition of C1 esterase cleavage of artificial substrates like N-acetyl L-tyrosine-ethyl ester which is a colorimetric method [15,16] and is a non-competitive mechanism for which the Michaelis-Menten constant, Km, is 0.017 mol/l at 37°C, in the optimum pH range 7.2-7.4 [16]. This kinetic constant for the substrate is dependent on the pH of the solution which indicates that a terminal amino group may be involved in catalysis [17].

The rhC1s diluted to 10 µg/ml in assay buffer was used to prepare a curve of C1-INH reference standard in assay buffer with concentrations of 0.01 to 20 nM. Equal volumes of diluted rhC1s and reference standard or purified C1-INH at different concentrations were mixed. Two rhC1s blanks containing assay buffer were included. The mixtures were incubated at room temperature for 30 min. The reaction was started by adding 50 µl of 200 µM substrate/DTNB mixture. The absorbance was read at 405 nm in kinetic mode for every 5-10 min on MULTISKAN EX (Thermo scientific).

Results and Discussion

DEAE Sepharose step to capture C1-INH

Filtered plasma was loaded on to the packed DEAE Sepharose column which was equilibrated with Sodium citrate pH 7.0 buffer. 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. The fraction containing the C1-INH protein was eluted with the buffer of higher conductivity (DEAE Chromatogram (Figure 1a). The column was washed with 0.5 M NaCl to remove the tightly bound proteins and 0.5 M NaOH for regeneration and sanitization. SDS-PAGE analysis and western blotting was performed for the column fractions to track for C1-INH (Figures 1b and 1c). Western blot analysis confirmed the presence of C1-INH in elution-1 peak and SDS-PAGE picture indicated C1-INH protein band with less intensity in elution-1 peak and its electrophoretic mobility was matching to the reference standard.

Cellufine phenyl step

The DEAE-Sepharose elution-1 peak with other impurities was loaded on to Cellufine phenyl column after adjusting the conductivity of sample equal to 0.8 M $(NH_4)_2SO_4$. The column was equilibrated with pH 7.0 buffer containing 0.8 M $(NH_4)_2SO_4$. The sample after adjusting the conductivity was loaded on to the equilibrated column at 90 cm/hr flow rate and under the applied conditions C1-INH did not bind to the column and was collected in the Flow through. The remaining impurities were eluted in the column regeneration steps (Figure 2a). The flow through sample was concentrated and diafiltered into 25

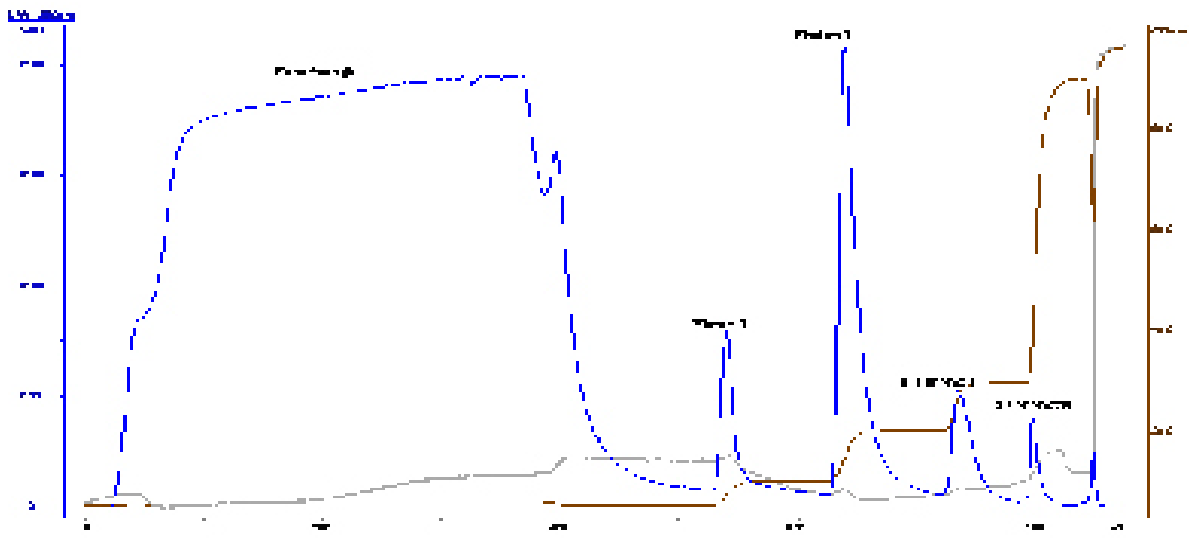


Figure 1a: DEAE Sepharose FF chromatography (1×10 cm, 20 ml) EQB: 20 mM sodium citrate pH: 7.0, Elution buffers: EQB containing NaCl. Eluate-1 contains C1-INH protein along with some impurities.Eluate-2 has other plasma proteins.

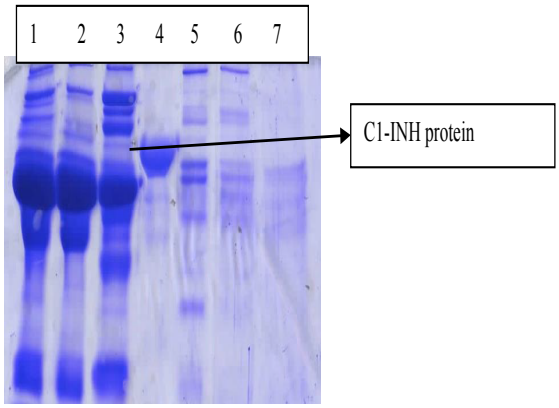


Figure 1b: SDS-PAGE analysis was performed to analyze the DEAE fractions. Samples were prepared in loading buffer containing 60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue and run on a 10% polyacrylamide gel. Lane 1: Load sample, 40 µg, + βME; Lane 2: Flow through, 40 µg, +βME; Lane 3: Elution-1 peak sample, 40 µg, +βME; Lane 4: Ref. standard, 20 µg, +βME; Lane 5: Elution-2 peak sample, 40 µg, +βME; Lane 6: 0.5M NaCl wash, 40µg, +βME; Lane 7: 0.5M NaOH peak, 40 µg, +βME; C1-INH protein can be seen in elution-1 fraction, a less intense band whose electrophoretic mobility matched the reference standard in lane 4.

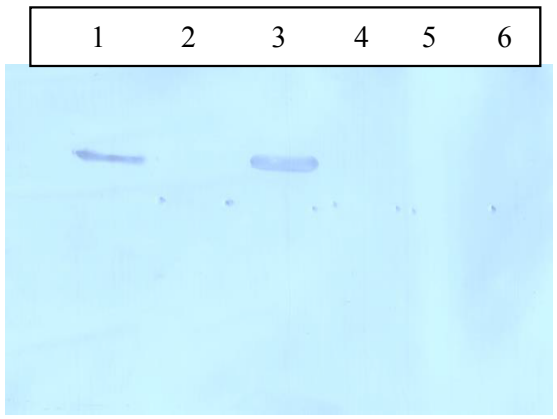


Figure 1c: Western blot analysis was performed to identify the fraction containing C1-INH protein. Samples were prepared in loading buffer containing 60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue and run on a 10% polyacrylamide gel and the gel is transferred on to a blot. Lane 1: DEAE load, 40 µg, +βME; Lane 2: Flow through, 40 µg, +βME; Lane 3: Elution-1 peak sample, 40 µg, +βME; Lane 4: Elution-2 peak sample, 40 µg, +βME; Lane 5: 0.5M NaCl wash, 40 µg, +βME; Lane 6: 0.5M NaOH peak, 40 µg, +βME; the protein of interest C1-INH was identified in load and elution-1 fraction.

mM sodium citrate pH 7.0 buffer using Amicon stirred cell loaded with 10 KDa molecular weight cut off membrane. This diafiltered sample was analyzed on SDS-PAGE. The C1-INH band was enriched in intensity but it was also found to have two more extra bands which are low molecular weight impurities (Figure 2b). In order to purify this further to homogeneity, the diafiltered sample was processed on TMAE column.

TMAE step to purify C1-INH to homogeneity

The diafiltered sample was loaded onto TMAE column at a flow rate of 90 cm/hr and after loading the column was washed with equilibration buffer to remove any unbound proteins. C1-INH bound to the column was eluted by changing the pH and ionic strength of the buffer. The bound impurities were removed by washing the column with 0.5 M NaCl and 0.5 M NaOH solutions (Figure 3a). All the fractions from the column were concentrated on a stirred cell in order to analyze on SDS-PAGE and the analysis indicated that the low molecular weight impurities did not bind to the column under the applied conditions where as C1-INH had bound to the column. The sample from the elution peak showed a single band on SDS-PAGE (Figure 3b).

The purified C1-esterase inhibitor was pooled, concentrated and subsequently exchanged into 10 mM sodium citrate buffer pH 6.8, containing 0.13 M Glycine and 0.14 M sodium chloride. This buffer exchange was carried out using 10 KDa TFF cassettes on a mini Pellicon TFF system. The observed yield was 100-120 mg of C1-INH per liter of human plasma by adapting the developed process and the purified molecule was characterized by the methods described in the following section.

SDS-PAGE analysis of the purified C1-INH

The product obtained after the TMAE step is typically a single chain protein which was found to be more than 98% pure as shown by SDS-polyacrylamide gel electrophoresis (Figure 4). On comparison with reference standard its molecular weight is 85-93 KDa under reducing conditions and 90-105 KDa under non-reducing conditions and the mobility matched that of the reference standard.

SE-HPLC analysis of the purified C1-INH

The identity and purity check by SE-HPLC analysis in comparison to standard indicated that the purified C1-INH protein is more than 98% pure (Figure 5). 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 C1-INH peak (15.827 min) closely matched the retention time of the reference standard (15.820 min) and also showed a better purity profile (98.9%) with a major single peak and a very minor peak at 11.779 min, when compared to the reference standard (92.4% purity) with at least two other contaminants on SE-HPLC chromatogram. Monomer along with the aggregate peak would be >95% purity for C1-INH reference standard. Rapid reactivation of C1INH-sample with polymers and dimers to functional monomers is possible by denaturation, on-column refolding method developed by Mathew Gauthier and Philip A. Patston [18].

RP-HPLC analysis of the purified C1-INH

RP-HPLC analysis indicated that the purified C1-INH protein is more than 99% pure (Figure 6). The analysis was carried out on Inertsil WP 300 C4 column (4.6 mm I.D×250 mm length) with particle size of 5 µm and the applied flow rate was 1 ml/min. Mobile phase A: 0.1% TFA in 10% Acetonitrile, Mobile phase B: 0.1% TFA in 90% Acetonitrile. The retention time of the purified C1-INH peak (28.50 min) also showed a good purity profile (99.43%) with a major single peak and two very minor peaks before the main peak on RP-HPLC chromatogram.-

Specific activity

Specific activity was estimated by the colorimetric method and the IC_{50} value was determined by plotting the OD values obtained for C1-INH reference standard and the purified C1-INH sample using Graph Pad prism software (Figure 7). The IC_{50} of the purified C1-INH protein (1.367) was comparable in value to that of the reference standard (1.392).

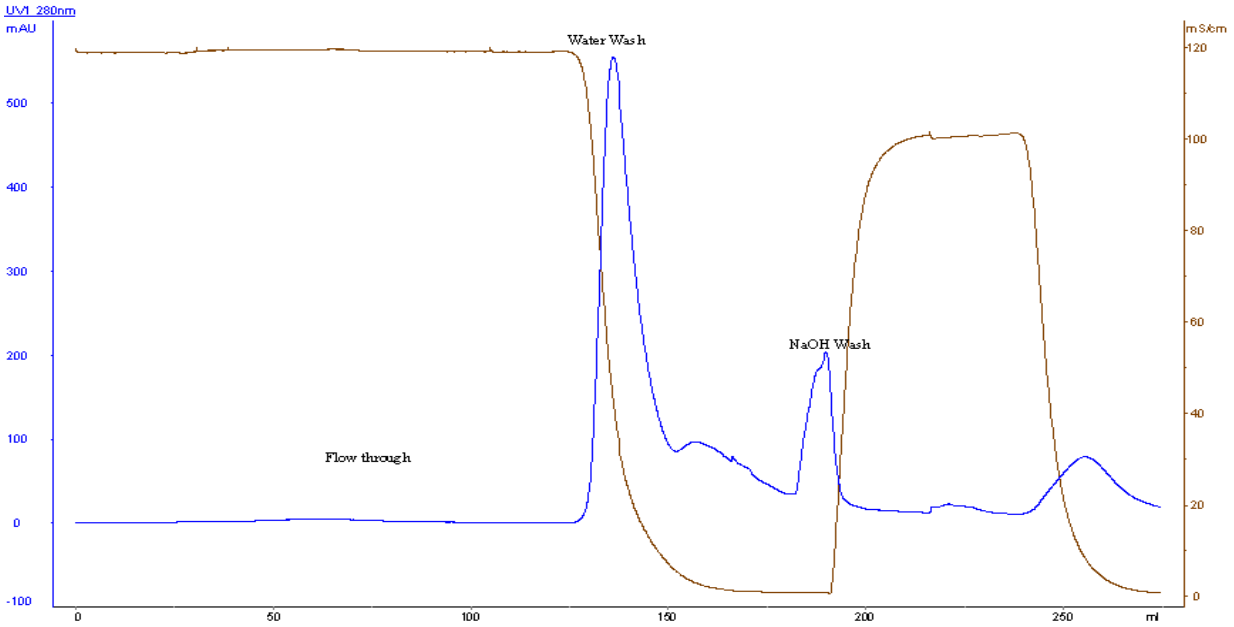


Figure 2a: Cellufine phenyl chromatography for the DEAE elution-1 fraction on a 20 ml column. Unbound C1-INH protein comes in flow through.

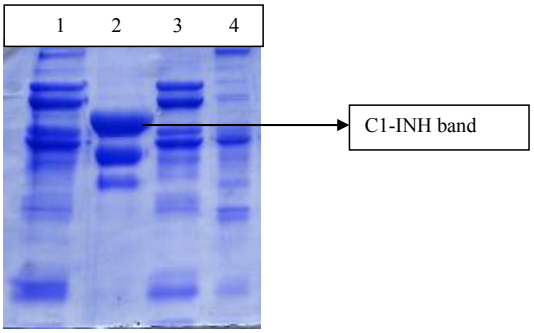


Figure 2b: SDS-PAGE analysis was performed to analyze the Cellufine phenyl column fractions. Samples were prepared in loading buffer containing 60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue and run on a 10% polyacrylamide gel. Lane 1: Load sample, 40 µg, +βME; Lane 2: Flow through (Concentrated and diafiltered) 40 µg, +βME; Lane 3: Water wash peak 40 µg, +βME; Lane 4: NaOH peak, 40 µg, +βME.

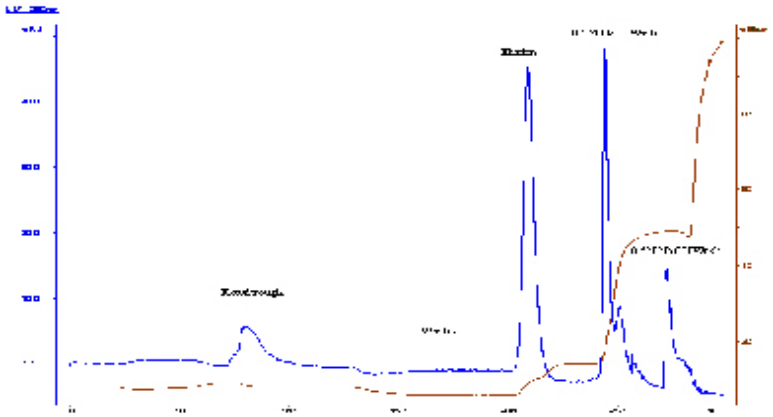


Figure 3a: TMAE chromatography for the Cellufine phenyl flow through on a (1×10 cm, 20 ml) column. EQB: 25 mM sodium citrate containing 30mM NaCl, pH 6.0, conductivity 8 mS/cm.Wash-2: 25 mM sodium citrate pH: 7.0, Elution buffer: 25 mM sodium citrate containing 170mM NaCl, Conductivity 20 mS/cm, pH: 7.0.

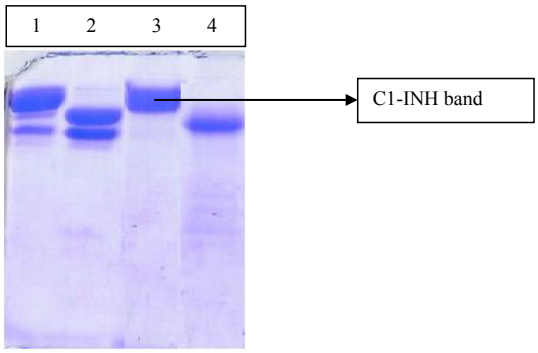


Figure 3b: SDS-PAGE analysis was performed to analyze the fractions from TMAE column. Samples were prepared in loading buffer containing 60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue and run on a 10% polyacrylamide gel. Lane 1: Column load sample, 40 µg, +βME; Lane 2: Column flow through 40 µg, +βME; Lane 3: Elution peak sample; Lane 4: 0.5M NaCl elution peak 40 µg, +βME.

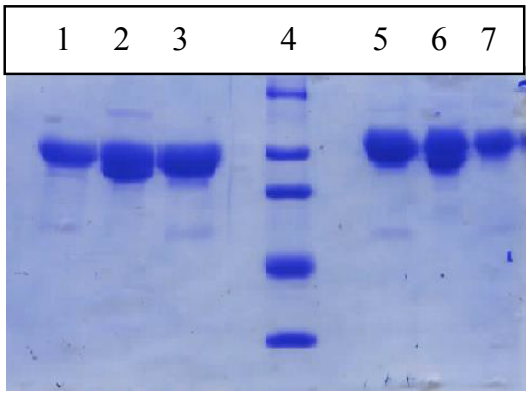


Figure 4: SDS-PAGE analysis was performed to check the purity of purified C1-INH protein in reference to C1-INH standard (R&D systems). Samples were prepared in loading buffer containing 60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue and run on a 10% polyacrylamide gel. Lane 1: Purified C1-INH, 10 µg, +βME; Lane 2: Reference standard 15 µg, +βME; Lane 3: Purified C1-INH, 15 µg, +βME; Lane 4: Molecular weight marker, 29-205 KDa; Lane 5: Purified C1-INH, 15 µg,-βME; Lane 6: Reference standard 15 µg,-βME; Lane 7: Purified C1-INH, 10 µg,-βME.

Conclusion

There are many reports of purification of C1-INH protein [4-6,8,10,12,13] and these previously published reports detail the usage of precipitation and affinity chromatography methods for purification of C1-INH and some of them have problem with low yields, time consuming process and does not result in pure protein of therapeutic grade. In the current research, an attempt was made to look for a purification scheme that is both economical and scalable for the purification of C1-esterase inhibitor, without compromising on yields, purity and its other therapeutic characteristics. The process developed was economical because it was devoid of multiple steps to purify C1-INH unlike some of the previous published reports which had-PEG precipitation, DEAE, Zinc chelate, Immuno adsorption steps and lysine Sepharose, DEAE Sephadex, Sephadex G-150, Hydroxy apatite chromatography . Some of the existing processes use affinity columns which are generally expensive. Besides an additional purification step will be required to remove the ligands as there is always a possibility of ligand leaching from the affinity column. But the current process does not require any affinity column and thereby eliminates the need

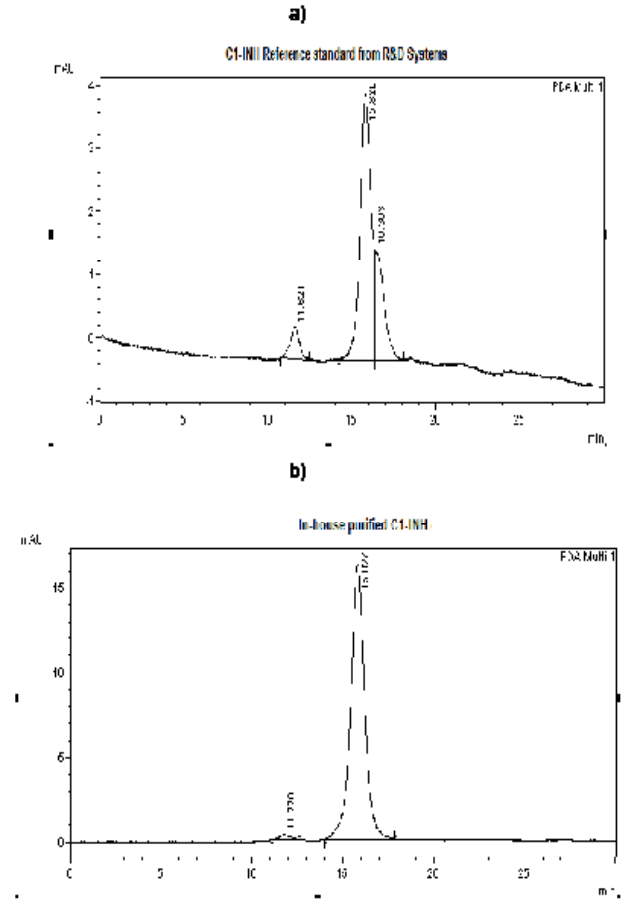


Figure 5: SE-HPLC analysis was performed to check the purity of purified C1-INH protein in reference to C1-INH standard shows that (a) Reference standard is 92.40% pure (b) In-house purified C1-INH molecule is 98.90% pure.

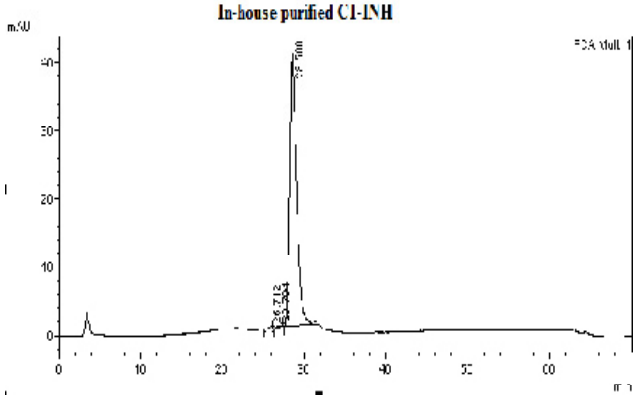


Figure 6: The above RP-HPLC chromatogram shows that the In-house purified C1-INH protein is 99.43% pure.

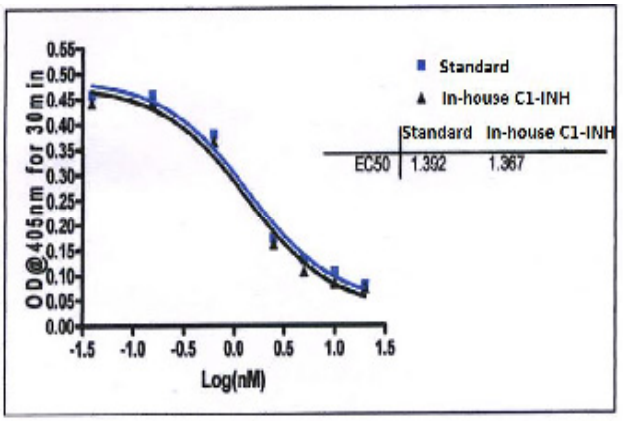


Figure 7: IC_{50} activity of the purified C1-INH molecule was estimated in reference to standard and the results indicated that it had similar activity to standard. O.D at 405nm was plotted versus log concentration using Graph Pad prism software.

for additional steps to remove leached ligands. Polyethylene glycol (PEG) was not used in the current process which brings down the cost of procuring PEG and also eliminates the usage of additional filters or equipments like centrifuge to separate the PEG precipitated protein from the solution. The developed process was optimized at 1 L scale and then scaled up to 10 L scale without any major challenges. This process was easily scalable as this was totally end to end chromatography based without any precipitation steps. Chromatography systems and columns are easily scalable from R&D to Production and the chromatography resins used in the process are also available in bulk quantities.

After an initial capture on DEAE resin, two different new generation resins were chosen for their ability to selectively bind and purify C1-INH from among the other plasma proteins. Figures 2a and 2b indicate how the use of Cellufine phenyl resin was able to purify C1-INH to a significant extent with the SDS-PAGE (Figure 2b) analysis revealing only a few protein impurities. As a polishing step for purity, the C1-INH sample after Cellufine phenyl 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 3b. The yield of the final purified C1-INH by this process was found to be in the range 100 to 120 mg/litre of human plasma. This increased recovery and purity helped us nail this as an ideal process scheme for therapeutic manufacturing, subject to the other biochemical characteristics being within the required specifications. Results of the characterization studies on purified C1-INH indicated the comparability of the protein with an existing reference standard with respect to its purity on SDS-PAGE, RP-HPLC analysis and specific activity (Figures 4-7). The overlap of reference protein standard curve with the purified C1-INH curve and their comparable IC_{50} values 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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