CHAPTER 7

IMPROVEMENT OF PURIFICATION STRATEGIES TO ACHIEVE HIGH RECOVERY YIELD, PURITY AND BIOLOGICAL ACTIVITY OF rhAT III PROTEIN FOR THEIR POSSIBLE THERAPEUTIC POTENTIAL
ABSTRACT

Antithrombin III (AT III) is a plasma glycoprotein that reduces the numerous physiological target enzymes of coagulation system. The effect of purification strategies plays an essential role in getting highest recovery yield, purity and biological activity of recombinant human antithrombin III (rhAT III). In the current research, the task of purifying rhAT III protein from *Saccharomyces cerevisiae* (*S.cerevisiae*) BY4741 was carried out using two different approaches such as cross flow filtration (CFF) system and chromatographic methods. In the first approach, the protein was concentrated and partially purified through CFF to achieve maximum yield of recovery and purity of 87 % and 94 %, respectively. In the next approach, purification process involved a single step chromatographic method with a range of ion exchange and size exclusion resins to evaluate the maximum recovery yield and purity of rhAT III. From the experimental results, it has been observed that the size exclusion chromatography (SEC) method with superose 12 matrix was appropriate for the purification of rhAT III and achieved the maximum yield of recovery and purity of 51 % and 97 %, respectively. Further, to obtain a high recovery yield and purity of rhAT III, the effect of different chromatographic conditions such as mobile phase, mobile phase pH, flow rate, sample volume and protein concentration were also studied. Under the optimal chromatographic conditions (superose 12; phosphate buffer with pH: 8.5; flow rate: 1mL/min; sample volume: 2 mL and sample concentration: 2 mg /mL), rhAT III protein was considerably recovered and purified in a single step with maximum recovery yield, purity and biological activity of 67 %, 99 % and 410 IU/L, respectively. Based on this research, it was concluded that size exclusion chromatography with superose 12 matrix was a more suitable and a potential technique for the purification of rhAT III.

7.1. INTRODUCTION

Antithrombin III (AT III) is a plasma glycoprotein (432 amino acids and 58 kDa) and is a member of serine protease inhibitor (SERPIN) family. AT III is also an important physiological regulator of different clotting factors, including thrombin, factor IXa, Xa, XIa, and XIIa [222 and 189]. The thrombin inactivation with AT III under common physiological conditions is slow, but the rate of complex formation increases in a high extent by heparin cofactor (HC) activity [223]. AT III has a therapeutic value for those who suffer from venous thrombosis and pulmonary
embolism with acquired or inherited inadequacy of AT III activity [224]. In these situations, an enough supply of pure AT III is necessary.

Though AT III is obtainable from human plasma [167 and 225], from the view of product safety and possible virus infection (retrovirus and hepatotropic viruses), AT III production must be done only through recombinant DNA (rDNA) tools [226]. rhAT III has been produced with different expression host systems (E. coli cells, Pichia pastoris cells, Chinese hamster ovary cells, COS1 monkey kidney cells, baby hamster kidney cells, Spodoptera frugiperda insect cells and transgenic goat milk) [145, 146, 227, 228, 229 and 230], but yeast-based rhAT III production has been exhibited only through Broker et al., [154].

To have the ability to separate a desired protein from a mixture, the physicochemical properties of the target protein should be used. Till now, there is no single or simple process to purify all kinds of proteins. On the other hand, if the recombinant based protein is intended for clinical use, it ought to be very pure. But, as considered to be in economic view, purification should be done in a single step.

The main objective of a purification process is not only the exclusion of undesired contaminants, but also the purification of the target protein [231]. Ion exchange chromatography (IEC) is certainly the most commonly used method for the partition and purification of proteins, polypeptides, poly nucleotides, nucleic acids and additional bio molecules. The reasons behind the achievements of IEC are its determining power, high ability, wide relevance, and the simplicity of the system [232]. On the other hand, SEC is also one more purification method in which the components are filtered according to their size, and some times in molecular mass. It is frequently used to purify the larger or macro molecules such as proteins and industrial polymers [233].

In the present study, purification of rhAT III from Saccharomyces cerevisiae BY4741 has been carried out using two different approaches such as cross flow filtration (CFF) and chromatography methods (IEC and SEC) to achieve high recovery yield and purity. In addition, the effects of various physicochemical parameters such as mobile phase, mobile phase pH, flow rate, sample volume and sample concentration on rhAT III purification were also investigated.
7.2. MATERIALS AND METHODS

The complete materials used for rhAT III purification is explained in Chapter 3. The methods for fed batch fermentation, cell lysis analysis, filtration efficiency through CFF, chromatographic purification, RP-HPLC, SDS-PAGE analysis, Western blotting and biological activity are also explained in the materials and methods part (Chapter 3). Fig. 7.1 shows the complete process flow chart for purification of rhAT III.
Fed batch fermentation

Cell lysis

CFF

Concentration and partial purification

FPLC

Purification strategies of rhAT III

Single step purification

Effect of chromatographic resin on rhAT

Effect of mobile phase on rhAT III

Effect of mobile phase $p^H$ on rhAT III

Effect of flow rate on rhAT III

Effect of sample volume on rhAT III

Effect of protein concentration on rhAT

Optimized chromatographic conditions

Characterization of rhAT III through RP-HPLC, SDS-PAGE and Western blot

Biological activity of rhAT III

Fig. 7.1 Complete process flow sheet for rhAT III purification through different purification strategies.
7.3. RESULTS AND DISCUSSION

7.3.1. Preparation of concentrated solution and fractional purification of rhAT

Cell lysed sample was concentrated and moderately purified through cross flow filtration system (CFF) using 30 kDa cartridge. The rhAT III filtration performance is shown in Fig. 7.2. From the experimental results, the change in rhAT III concentrations (0.4, 1.1, 2.6, 3.9, 5.7, 6.8 and 7.2 mg/mL) with change in flux (21, 17.5, 13.6, 10.8, 6.7, 3.5 and 2.9) was observed (Fig.7.2) at progressive time periods (0, 30, 60, 90, 120, 150 and 180 minutes). It can be clearly observed that a noteworthy increase in protein concentration has been observed with decrease in flux as a function of time.

At a specific time period (180 min), no significant change was observed and a high 7.2 mg/mL of rhAT III concentration was obtained with a constant flux of 2.9 mL/min. This effect may be due to the clogging of pores by the formation of foam on the surroundings of membrane that constrained the filtration of solution from the membrane. The filtration was ended after recovering 40 mL retentate with a concentration factor of 12.5 and retention factor of 0.87. The rhAT III recovery yield and purity after the filtration step was about 87% and 94% respectively. Various recombinant proteins such as recombinant human granulocyte stimulating factor (rhG-CSF) and monoclonal antibodies have been successfully concentrated with cross flow filtration system and it was achieved with > 95% purity [169 and 218].
Fig. 7.2 Clarification and concentration of rhAT III. The rhAT III filtration performance is shown during ultra filtration with hollow fiber cartridge. rhAT III concentration was shown increasing with respective to the decrease in flux as a function of time; (■) rhAT III concentration; (♦) flux

7.3.2. The effect of physicochemical parameters on rhAT III purification

Optimization should evaluate a number of physicochemical parameters including stationary and mobile phase for effective protein purification. Hence, in this work, a study has been carried out on the stationary phase, mobile phase, mobile phase pH, sample flow rate, injection volume and mass load to analyze and evaluate the effect of yield and purity of rhAT III.

7.3.2.1. The effect of chromatographic resin on rhAT III purification

Optimization should evaluate a number of physicochemical parameters including stationary and mobile phase for effective protein purification. Therefore, the studies have been conducted on rhAT III purification to analyze the influence of ion exchange and size exclusion chromatographic resins. The recovery yield and purity of rhAT III for both ion exchange and size exclusion chromatography with different resins are shown in Table 7.1. From the results, it can be seen that among various resins selected in both IEC and SEC, SEC with Superose 12 showed maximum recovery yield and purity of 51 % and 97 % respectively. The lower yields with ion
exchange could be attributed to low permeability and capacities of resins for larger proteins such as AT III. Additionally, the hydrophobic character of the ion exchange resin will denature the proteins [234]. Therefore, further experiments were carried out with Superose 12. The above experimental results attained in the current work were significantly superior to the previously reported work by Mak et al. [235]. The effect of various chromatographic resins on recombinant protein (rhG-CSF) purification was also successfully studied by other researchers [105].
Table 7.1 The effect of stationary phase on rhAT III purification. Maximum recovery yield (51 %) and purity (97 %) of rhAT III is shown with Superose 12. The experimental result analysis represents mean values with standard variation (SV) of three (triplicates) repeated trials.

<table>
<thead>
<tr>
<th></th>
<th>Ion exchange resin</th>
<th>Size exclusion resin</th>
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<tr>
<td></td>
<td>Q-Sephrose</td>
<td>Capto Q</td>
</tr>
<tr>
<td></td>
<td>CM Sepharose FF</td>
<td>Sephacryl S200</td>
</tr>
<tr>
<td></td>
<td>Sephadex G-100</td>
<td>Superose 12</td>
</tr>
<tr>
<td>Recovery yield (%)</td>
<td>43 ± 1.2</td>
<td>46 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>48 ± 1.1</td>
<td>49 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>48 ± 1.2</td>
<td>48 ± 1.2</td>
</tr>
<tr>
<td>Purity (%)</td>
<td>96</td>
<td>96</td>
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<tr>
<td></td>
<td>97</td>
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7.3.2.2. The effect of mobile phase on rhAT III purification

The choice of mobile phase plays a critical role in the purification of proteins because they ought to be adapted to the buffer atmosphere during the elution. In the present work, a study has been performed on four different mobile phases (tris, acetate, phosphate and sulphate) with pH 8 using size exclusion chromatography with Superose 12.

The recovery yield and purity of rhAT III with four different mobile phase buffers were shown in Table 7.2. From the experimental results, recovery yields (50, 52, 54 and 51 %) and purities (97, 98, 98 and 97 %) of rhAT III were obtained with respective mobile phase buffers such as tris, acetate, phosphate and sulphate buffers. From the above result analysis, it was concluded that phosphate buffer has achieved a high recovery yield (54 %) with 98 % purity. The lowest recovery yield (50 %) was achieved with Tris buffer with 97 % purity. However, acetate and sulphate are next to the phosphate with recovery yields of 52 and 51 % respectively. The results attained in the present research were superior to the previously reported work [236]. The effect of mobile phase on recombinant protein (rhG-CSF) purification was also successfully studied by other researchers [169].

Table 7.2 The effect of mobile phase on rhAT III purification. The maximum recovery yield (54 %) and purity (98 %) of rhAT III is shown with Superose 12 matrix. The experimental result analyses indicate mean values with standard variation (SV) of three (triplicates) repeated trials.

<table>
<thead>
<tr>
<th>Mobile phase Buffer</th>
<th>Recovery yield (%)</th>
<th>Purity (%)</th>
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<tbody>
<tr>
<td>Tris</td>
<td>50 ± 1.1</td>
<td>97</td>
</tr>
<tr>
<td>Acetate</td>
<td>52 ± 1.2</td>
<td>98</td>
</tr>
<tr>
<td>Phosphate</td>
<td>54 ± 1.1</td>
<td>98</td>
</tr>
<tr>
<td>Sulphate</td>
<td>51 ± 1.1</td>
<td>97</td>
</tr>
</tbody>
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7.3.2.3. The effect of mobile phase pH on rhAT III purification

Purification of proteins is pH dependent, which can definitely influence the recovery yield and purity [106]. In the present research, a study has been carried out on phosphate buffer with different pH values ranging from 6.5 to 9.0 to estimate their
effect on recovery yield and purity of rhAT III. The recovery yield and purity of rhAT III at various phosphate buffer pH values are shown in Fig.7.3.

From the experimental results, recovery yields (48, 50, 51, 53, 55 and 52 %) and purities (97, 97, 98, 98 and 98 %) of rhAT III were obtained with respective mobile phase pH such as 6.5, 7, 7.5, 8, 8.5 and 9.0. It has been clearly observed that the maximum recovery yield (55 %) and purity (98 %) of rhAT III was observed with buffer at pH 8.5. The lowest recovery yield (48 %) and purity (97 %) of rhAT III was observed with buffer at pH 6.5. Even though the purity was high (98 %) at pH 7.5, 8 and 9, low recovery yields (51, 53 and 52 %) were observed when compared to pH 8.5. The mobile phase pH on recombinant protein purification was also successfully analyzed by a previously reported work [169].

Fig.7.3 The effect of mobile pH on rhAT III purification. The maximum recovery yield (55 %) and purity (98 %) of rhAT III was shown with pH 8.5. Filled diamond represents recovery yield; Filled square represents purity. The experimental result analyses indicate mean values with standard variation (SV) of repetitive (duplicates) trials.
7.3.2.4. The effect of flow rate on rhAT III purification

Flow rate should be considered as one of the most significant parameters during protein purification because in size based separations it may influence the protein structure, biological activity and recovery yield. In the present work, a study has been conducted with different flow rates of 0.5, 1.0, 1.5 and 2.0 mL/min to show the impact of flow rate on rhAT III purification. The recovery yield and purity of rhAT III at different flow rates are shown in Fig.7.4. From the experimental results, recovery yields (59, 66, 61 and 57 %) and purities (98, 99, 98 and 98 %) of rhAT III were obtained with respective flow rates such as 0.5, 1, 1.5 and 2 mL/min flow rate. It can be clearly seen that the high recovery yield (66 %) and purity (99 %) of rhAT III was obtained with 1mL/min flow rate. Although using lower sample flow rates results in longer run times, the increased resolution gives greater confidence and optimum column efficiency [237]. The low recovery yield (57 %) was observed with 2 mL/min due to high acceleration, which increased the chance of escaping and decreased the passage of the sample from the resin resulting in low recovery yields. Though the purity of rhAT III is similar at 0.5, 1.5 and 2 flow rates, variations in recovery yields (59, 61 and 57 %) were observed. Hence further experiments were carried out with 1.0 mL/min flow rate. The results obtained in this study are very close to the previously reported work [238].
Fig. 7.4 The effect of flow rate on rhAT III purification. The maximum recovery yield (66%) and purity (99%) of rhAT III was shown with 1 mL/min flow rate. *Filled diamond* represents recovery yield; *filled square* represents purity. The experimental result analyses indicate mean values with standard variation (SV) of repetitive (duplicates) trials.

7.3.2.5. The effect of sample volume on rhAT III purification

Sample injection volume can influence the protein purification and it affects mostly recovery yields. Therefore, in the present study, several experiments were carried out on rhAT III purification with different sample volumes such as 1, 2, 3, 4 and 5 mL. The recovery yield and purity profiles of rhAT III with varying sample volumes are shown in Fig. 7.5. From the experimental results, recovery yields (55, 60, 57, 56 and 58%) and purities (98, 98, 98, 98 and 98%) of rhAT III were obtained with respective sample volumes such as 1, 2, 3, 4 and 5 mL. From result analysis, it was concluded that the maximum recovery yield (60%) and purity (98%) with the sample volume of 2 mL were observed. This is attributed to the suitable volume of the sample that enters through the resin and increases the passage of desired protein molecules. The lowest recovery yields of 55 and 56% were achieved with 1 and 4 mL volumes respectively. This is due to the broadening of sample zone by increasing the sample volume and decreasing the passage of the sample through the resin. Therefore, further studies were continued with 2 mL sample volume.
Fig. 7.5 The effect of sample volume on rhAT III purification. The maximum recovery yield (60 %) and purity (98 %) of rhAT III was achieved with 2 mL sample volume. Filled diamond represents recovery yield; filled square represents purity. The experimental result analyses indicate mean values with standard variation (SV) of repetitive (duplicates) trials.

7.3.2.6. The effect of protein concentration on rhAT III purification

Sample concentration may also influence the chromatographic resolution and sensitivity [107]. In the present work, a study has been conducted on different protein concentrations ranging from 1 to 4 mg/ml with Superose 12 matrix to analyze the protein concentration effect on recovery yield and purity of rhAT III. Fig. 7.6 shows the recovery and purity of rhAT III under various sample concentrations. From the experimental results, recovery yields (63, 67, 61 and 60 %) and purities (98, 99, 99 and 98 %) of rhAT III were obtained with respective protein concentrations such as 1, 2, 3 and 4 mg/mL. From the above result analysis, it can be clearly seen that the high recovery yield (67 %) and purity (98 %) of rhAT III were achieved with protein concentration of 2 mg/mL. Even though a high purity (98 %) of rhAT III was observed with protein concentration of 3 mg/mL, a low recovery yield of 61 % was recorded. From this study, it was concluded that the loss of recovery yields could be
observed with increase in protein concentration due to non-binding interactions of protein with resin.

Fig.7.6 The effect of protein concentration on rhAT III purification. The maximum recovery yield (67 %) and purity (99 %) of rhAT III was shown with 2 mg/mL concentration. Filled diamond represents recovery yield; filled square represents purity. The experimental result analyses indicate mean values with standard variation (SV) of repetitive (duplicates) trials.

7.3.3. Optimal conditions for rhAT III purification

Based on previous experiments, the optimal chromatographic conditions were preferred for rhAT III purification: Superose 12 matrix, phosphate buffer at pH 8.5, flow rate 1mL/min, sample volume 2 mL and sample concentration is 2 mg/mL. Fig. 7.7 shows the FPLC chromatogram of rhAT III eluted under the aforesaid optimized conditions. It can be observed that the single major peak was observed and it indicates the monomeric form of rhAT III. The major peak appeared with high resolution at a retention volume of 13.3 mL indicating the active fraction of rhAT III. The whole chromatographic process was carried out in 25 min. The obtained recovery yield and purity of rhAT III under optimum conditions were 67 % and ≥ 98 %. The above experimental results achieved in the current work are significantly superior to the previously reported work [167 and 236] and obtained results are also very close to the earlier reported work [239].
Fig. 7.7 Fast protein liquid chromatogram of rhAT III (*) eluted from the size exclusion chromatographic (SEC) column. The maximum yield (67 %) and purity (99 %) of rhAT III is shown under optimum conditions: stationary phase: Superose 12; phosphate buffer with pH: 8.5; flow rate: 1mL/min; sample volume: 2 mL and sample concentration: 2 mg /mL.

Fig.7.8 shows the RP-HPLC chromatogram eluted from the SEC column under optimized conditions. It can be seen that the purity of rhAT III was shown to be 98 as similar with retention time (22.756 min) of standard rhAT III.
7.3.5. CHARACTERIZATION OF PURIFIED rhAT III

7.3.5.1. SDS-PAGE, Western blot and biological activity of rhAT III

The purity of rhAT III was also assessed through SDS-PAGE and western blotting. SDS–PAGE analysis of rhAT III was shown in Fig.7.9. A single target rhAT III band confirms the molecular mass of 58 kDa, which is similar to the control (standard rhAT III).

Fig.7.9 SDS-PAGE analysis of rhAT III. Lane M shows Marker; Lane 1 shows purified rhAT III; Lane 2 shows reference rhAT III. A single target band was observed with molecular weight of approximately 58 KDa and which is similar to the reference standard.
After that, the gel was transferred to PVDF membrane and the transformation was immuno detected with rhAT III antibodies and a single rhAT III band is shown in Fig.7.10 which is analogous to the reference standard. The maximum purified rhAT III biological activity was (410 IU/L) measured as heparin cofactor (HC) activity.

![Western blot analysis of rhAT III](image)

Fig.7.10 Western blot analysis of rhAT III. Both purified and reference standard rhAT III bands were characterized using polyclonal antibody and HRPO conjugated goat IgG antibodies against rhAT III.

7.4. CONCLUSION

In conclusion, SEC is found to be an effective technique for rhAT III purification and showed a maximum recovery yield (51 %) and purity (97 %) when compared to the IEC recovery yield (48 %) and purity (97 %). Further investigations on physicochemical chromatographic conditions such as mobile phase, mobile phase pH, flow rate, sample volume and sample concentration also proved their influence on recovery yield and purity. Under the optimized conditions, the maximum recovery yield, purity and biological activity of rhAT III was found to be 67 %, 99 % and 410 IU/L, respectively. The purity was evaluated through various analytical techniques such as RP-HPLC, SDS-PAGE and western blotting.