CHAPTER 4

CLONING AND EXPRESSION STUDIES OF AT III PROTEIN IN SACCHAROMYCES CEREVISIAE BY4741
ABSTRACT

Recombinant protein drugs have several advantages when compared with the traditional medicines such as low toxicity, high activity, specificity, clear biological function as well as facilitation of clinical application and mass production. The intention of this research work was to express and purify recombinant human antithrombin III (rhAT III) in Saccharomyces cerevisiae (S.cerevisiae) BY4741 expression host system. Human antithrombin III gene was amplified through specific primers and cloned into pYES2/CT vector with His-tag for easier purification with Ni\(^{2+}\)-chromatography. Further, the rhAT III was purified to homogeneity and was confirmed with SDS-PAGE and western blotting analyses. rhAT III was also characterized through two dimensional (2D) electrophoresis. Finally, biological activity of rhAT III was performed through hemoagglutination inhibition assay.

4.1. INTRODUCTION

Antithrombin III (AT III) plays an important role in maintaining the flow of blood. Blood coagulation system is mediated by a series of serine proteases. AT III is a potent inhibitor of Factors IXa, Xa [179], XI [180] and Xlla [181]. AT III is produced in the liver and has a plasma level of approximately 150 mg/L with molecular weight of 58 KDa [1]. The primary amino acid structure of human AT III had been completely determined by Petersen et al., and it was reported that the rhAT III protein has approximately 430 amino acid residues, 4 glucosamine-based oligosaccharide side units, and 3 disulfide bridges [182]. Serine protease inhibitors (serpins) represent a family of homologous proteins [183]. The comparison between the members of this group was first reported for alpha-AT, AT III and ovalbumin [184].

Transient Ischemic Attack, Thrombosis, Thrombocytosis and Cerebrovascular diseases are the leading causes of death and disability around the globe. Blood clot dissolving agents that are used to dissolve the thrombus have gained lot of importance in present day research [185]. Clinically approved clot-busting drugs like t-PA, Urokinase, Streptokinase, Tenecteplase and Reteplase have become most popular in recent times. Although these are in use for the patients who suffer from acute myocardial infection and stroke, some alternative thrombolytics are still in research phase [186]. Recombinant proteins have been expressed in E. coli strains [187] are far better characterized than any other microorganisms. AT III has been expressed by
rDNA technology using different expression systems like *E. coli*, Chinese hamster ovary cells and *Pichia pastoris*, but yeast derived rhAT III has been reported only by Broker et al. [154].

It is important to have a full understanding of the properties of the different *Escherichia coli* (*E.coli*) host strains in order to carry out molecular genetic techniques effectively. *E.coli* strains are frequently used for the propagation and recombinant DNA manipulation. It was originally chosen as a model system because of its ability to grow on chemically defined media and its rapid growth rate. Genotype indicates the genetic state of the DNA in an organism. It is associated with an observed behavior called the phenotype. Recombinant proteins such as growth factors are invaluable for therapeutic and diagnostic purposes. Utilising the robust and relatively low cost *Escherichia coli* expression system for recombinant protein production seems very promising. However, its production in *E.coli* suffers from several issues including poor solubility, low yield and inclusion bodies.

In the present research work, AT III was cloned and expressed in *S. cerevisiae* BY4741. The present work employs various strains, hosting compatible expression vectors, which are capable of selection and replication in both *E. coli* and yeast, particularly *S. cerevisiae* BY4741[188].

### 4.2. MATERIALS AND METHODS

The complete information about the materials used for the optimization of rhAT III was given in the materials and methods part (Chapter 3). The methods for expression and characterization of rhAT III (section 3.1) were explained in the materials and methods part (Chapter 3). Fig. 4.1 shows the complete flow chart for gene cloning, expression and characterization of rhAT III.
Fig. 4.1 The complete flow chart for gene cloning, expression and characterization of rhAT III
4.3. RESULTS AND DISCUSSION

4.3.1. Transformation analysis of bacterial strains

In the present work, transformation efficiency of clone (pGEM-T with AT III gene) (Genewiz Inc, USA) was compared through two different bacterial strains (JM109 and TOP10) through Hanahan methods of transformation to increase copy number of clone (pGEM-T with AT III gene) and its maintenance. Initially, the bacterial strains (JM109 and TOP10) were streaked on LB agar plates and a single colony from each bacterial strain plates were picked and grown in LB medium overnight at 37 °C with shaking conditions (120 rpm). Both the bacterial cultures (JM109 and TOP10) were again transferred to respective LB medium flasks (500 mL) and were incubated with vigorous shaking at 300 rpm and 37 °C till the cell density reached the designated absorbance at 600 nm (OD<sub>600</sub>). Then, both the bacterial cells (JM109 and TOP10) were collected through centrifugation (10,000 rpm and 10 minutes) and transferred to 10 % of ice-cold glycerol (15 mL) wash. Washed cells were again transferred to 10 % ice-cold glycerol and distributed into 100 µL aliquots with suitable number of cells estimated from the following formula

\[
OD_{600} = 5 \times 10^8 \text{ cells/mL}
\]  

(4.1)

The transformation efficiencies of both bacterial strains (JM109 and TOP10) represented by the number of transformants per microgram of DNA were shown in Fig.4.2. From the experimental results, it has been observed that maximum transformants/micrograms for TOP10 (1 X 10<sup>10</sup>) and JM109 (2X 10<sup>9</sup>) were estimated. It has also been observed that the transformation efficiency by the Hanahan protocol is low in JM109 compared to TOP10 cells. The reason for high transformation efficiency in case of TOP10 cells may be due to its good phenotypic characteristics.
Fig. 4.2 Transformation efficiency analysis of bacterial cells: pGEM-T with AT III gene construct (5 ng) was transformed to both JM109 and TOP 10 competent cells through chemical transformation. Maximum transformation efficiency ($1 \times 10^{10}$) was observed in TOP 10 cells when compared to JM109 cells ($2 \times 10^9$).

Further, experimental analysis was carried out with TOP10 *E. coli* cells to screen out the best transformation method through two different transformation methods (chemical and electroporation transformation method) for proper maintenance of clone. From the experimental result analysis, it has been observed that maximum transformation efficiency ($1.4 \times 10^{10}$) was observed through electroporation method when compared to chemical transformation method ($1 \times 10^{10}$) (Fig. 4.3).
Fig.4.3 Transformation efficiency analysis of pGEM-T along with AT gene constructs in Top 10 cells through chemical and electroporation transformation methods. Maximum transformation efficiency ($1.4 \times 10^{10}$) was observed through electroporation method when compared to chemical transformation ($1 \times 10^{10}$) methods.

4.3.2. Plasmid and cloning analysis

In the present work, pGEM-T along with AT III gene (Genewiz, USA) was transformed into TOP10 *E.coli* cells for proper maintenance of clone. After transformation of clone (pGEM-T with AT III gene) into TOP10 *E.coli* cells, again the clone (pGEM-T with AT III gene) was isolated from TOP10 *E.coli* cells and 10 µL of the purified plasmid was loaded on 1 % agarose gel along with marker for conformation of pGEM-T along with AT III gene molecular weight (MW). Fig.4.4 shows the molecular weight of purified pGEM-T along with AT III gene (lane 1 and 2). From the experimental results, it has been observed that the molecular weight of pGEM-T along with AT III gene was about 4.5 kb which was compared with MW marker (fermentas).

Fig.4.4 pGEM-T with AT III gene was isolated from overnight TOP10 culture and analyzed by 1 % agarose gel electrophoresis. Lane 1 and 2 show purified plasmid with molecular weight of approximately 4.5 kbp and compared with molecular weight marker (Lane M).
4.3.3. Amplification analysis of AT III cDNA target

After confirmation of molecular weight of pGEM-T along with AT III gene, the clone was subjected to PCR amplification using specific forward and reverse primers. The amplified AT III cDNA was again loaded onto 1 % agarose gel for conformation of its molecular weight. From the experimental result analysis, it has been observed that the molecular weight (MW) of AT III gene was 1425 bp which was compared with the MW marker (Fig.4.5).

![Fig.4.5](image)

**Fig.4.5** Samples of the PCR products were analysed through 1.2 % agarose gel electrophoresis and stained with ethidium bromide. Lane M shows size markers of a DNA ladder, lanes 1 and 2 show the position of migration of the antithrombin III product (predicted size, 1425 bp)

4.3.4. Digestion analysis of pYES2/CT gene constructs

The amplified AT III gene was then inserted into pYES2/CT vector using restriction enzymes (EcoRI and XhoI). After insertion of AT III gene into a pYES2/CT vector, again the clone was digested through EcoRI and XhoI restriction enzymes for further conformation of both vector (pYES2/CT) and AT III gene molecular weight through 1 % agarose gel electrophoresis. Typically, analytical digests were carried out in a total volume of 20 µL containing 5 µg of plasmid DNA. Digests for subcloning applications were typically carried out in a 50 µL volume containing 25 µL of plasmid DNA. 5 µL aliquots of the reaction mixture were taken every hour until complete digestion could be visualised after agarose gel electrophoresis. 10 µL of undigest DNA and double digest was loaded to check the
digested pattern along with DNA marker to confirm its molecular weight. Fig. 4.6 shows the undigest (lane 1) and double digested DNA (lane 2) sample along with marker (lane M). From the experimental results, the molecular weight of pYES2/CT and AT III gene after restriction digestion analysis were clearly observed. Hence, further analyses were carried out with pYES2/CT with AT III gene construct.

![Fig. 4.6](image)

**Fig. 4.6** Restriction digestion analysis of clone (pYES2/CT with AT III gene): Lane 1 shows Uncut DNA/Undigested DNA (pYES2); Lane 2 shows Digests of EcoRI and XhoI; Lane M shows Marker (Supermix DNA ladder, MCLAB products)

### 4.3.5. Purification analysis of rhAT III protein

After restriction digestion analysis of pYES2/CT along with AT III gene, the clone was transformed into *S. cerevisiae* BY4741 host system through electroporation transformation method. After transformation, a single colony of cells was inoculated into 100 mL YNB-URA def medium and kept for incubation and induced with 20% galactose and 10% raffinose. After reaching high cell density, the cells were subjected to sonication and the lysed cells were subjected to centrifugation. Then the protein sample was subjected to the affinity chromatography using a Nickel-Column for purification of rhAT III protein.

Fig. 4.7 shows the flow through (FW), wash fractions (W1, W2 and W3) and elution fractions (E1 and E2) of proteins. From the experimental result analysis, it has been clearly observed that other proteins of host system were also detected along with rhAT III in flow through (lane FT). The gel also showing the wash fractions (lane W1, W2 and W3) contained few similar bands along with rhAT III protein. It has also
been analyzed that both the eluted fractions (lane E1 and E2) showed a single band with a similar mobility as standard rhAT III (lane Ctrl) (NIBS, UK) and the molecular weight of rhAT III (58 kDa) fractions were also compared with marker (lane M). However, the other proteins of host system (*S. cerevisiae* BY4741) were not detected by rhAT III antibodies in western blot (Fig. 4.9).

**Fig.4.7 Analyses of rhAT III by SDS-PAGE.** His-tagged rhAT III was separated from *Saccharomyces cerevisiae* BY4741 culture media by binding with Ni-NTA His bind resin. Flow through (FT), washes (W1, W2 and W3) and elutions (E1 and E2) of rhAT III samples along with medium range molecular weight marker (Lane M) were analyzed using 12 % SDS-PAGE.

**4.3.6. Two dimensional (2D) gel electrophoresis analysis**

Two dimensional (2D) electrophoresis was carried out for the visualization of isomeric forms of rhAT III in the sample. Initially, gel was incubated in 30 % ethanol and 10 % glacial acetic acid for 30 min with two exchanges of the fixing solution (50 % methanol). Then the gels were washed three times with 2 % glacial acetic acid for 10 minutes each and balanced in prestaining buffer (12 % (*NH₄*)₂SO₄, 2 % glacial acetic acid, and 50 % ethanol) for another 30 minutes. Finally, the gel was stained in staining solution overnight until the spots of interests were manually picked. After the completion of the process, the gel was scanned on gel scanner with white light converter (UVI-Tech, Lark Innovative) and the resulting images were analyzed through image master software. Fig. 4.8 shows the 2D electrophoresis analysis of
rhAT III. In the experimental results, rhAT III with the isoelectric point of 4.5 to 5.5 was clearly observed.

![Image of 2D electrophoresis](image.png)

**Fig.4.8** Two-dimensional (2D) electrophoresis analysis of rhAT III. rhAT III separation using IEF on pH 4-7 strip and followed by 12% SDS-PAGE. After separation of rhAT III, gel was stained with eZee blue direct stainer solution.

### 4.3.7. Western blot analysis of rhAT III

In order to assess the immunological relationship between purified rhAT III protein and its AT III antibodies, the protein sample eluted from the affinity column was submitted to SDS-PAGE and blotted onto nitrocellulose membrane. Fig.4.9 shows the reactivity of purified rhAT III protein (from two different extracts) with the AT III antibody (Abcam, ab92621) and Goat antithrombin antibody (HRP) (Abcam, ab97080) at 1/5000 dilution. From the result analysis, it can be seen that the 58 kDa rhAT III protein band strongly reacted with AT III antibody indicating that the purified protein is rhAT III protein.
Fig.4.9 Western blot analysis of rhAT III from two separate purification runs. Approximately 20 µg of purified protein was loaded onto 12 % SDS-PAGE and transferred to nitrocellulose membranes, and western blot was carried out using ab92621 antibody against rhAT III protein.

4.3.8. Hemagglutination inhibition assay of rhAT III

In order to test the bioactivity of rhAT III, hemagglutination inhibition assay was performed using human peripheral RBC. In brief, 100 µL of RBCs fixed in 3 % glutaraldehyde were incubated with carbohydrate specific antibody (1:1000 dilution) and 0-10 mM of rhAT III in PBS. RBCs incubated through carbohydrate specific antibody alone were used as negative control (Fig.4.10). From the experimental result analysis, it has been observed that wells containing RBCs incubated with carbohydrate specific antibody and 1-10 mM of rhAT III showed sheet like agglutination. It has also been observed that wells containing RBCs incubated with carbohydrate specific antibody and 0 mM rhAT III (PBS control) and RBCs incubated with carbohydrate specific antibody alone (negative control) showed button like sedimentation.

Fig.4.10 Hemagglutination inhibition assay of rhAT III. Well 1-10: Sheet like agglutination was observed when a rhAT III is present. Well 0 and Control (C): Button like sedimentation was observed when rhAT III is absent
4.4. CONCLUSION

Apart from the thrombin inhibition and additional activated clotting factors, AT III may also have the capacity to modulate the cellular expression of proinflammatory cytokines (PIC) and procoagulant tissue factor (PTF), contributing to the rationale for its use in the treatment of sepsis. Nowadays, thrombolytic therapy needs an ideal drug against thrombosis. The recombinant therapeutic proteins produced through r-DNA technologies have more scope in the healing of thrombin related diseases. As developments in molecular cloning, rhAT III has been produced in soluble form in order to minimize drawback of native AT III from industrially feasible vector pYES2/CT. AT III is also one of the important anticoagulants in human plasma proteins. rhAT III expression through construction of yeast vector (pYES2/CT) containing human antithrombin cDNA sequence shows antithrombin activity.

Although it is of huge interest to get reliable, effective and simply obtainable purification techniques for AT III, the quest has been typically failed. However, finding rapid and competent method is of interest, but the search could advantage from looking past AT III. In this research, we inserted the AT III gene in pYES2/CT vector and transformed into Saccharomyces cerevisiae BY4741 for the production of rhAT III protein.

The rhAT III was expressed in Saccharomyces cerevisiae BY4741 by developing the yeast culture in YNB (-URA def) media and purified through nickel-based affinity chromatography because of its His 6 tag. On the other hand, in order to clarify whether rhAT III protein expression was controlled throughout improvement in yeast cells, western blot study was carried out. After cell lysis analysis, the rhAT III was recognized as a single 58 kDa band by 12 % SDS-PAGE and was further characterized with 2D gel electrophoresis and western blotting.

This indigenously produced rhAT III was evaluated for its biological activity using hemagglutination inhibition assay before being used with the original studies presented in this thesis. Still a lot of research has to be done to minimize the risks during thrombolytic therapy. In future, it will be an ideal drug for the patients who need thrombolytic therapy.