

EXPRESSION AND CHARACTERIZATION OF RECOMBINANT HUMAN ANTI-THROMBIN IN *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

Objective: The purpose of this study was to express and purify recombinant anti-thrombin in *Saccharomyces cerevisiae* BY4741 expression system.

Methods: cDNA of human anti-thrombin gene was amplified with specific primers and cloned into pYES2/CT vector with His-tag for easier purification using Ni²⁺-NTA chromatography.

Results: Protein was purified to homogeneity and was confirmed as anti-thrombin, using SDS-PAGE and western blotting. We characterized recombinant anti-thrombin and compared it with human plasma-derived anti-thrombin.

Conclusion: Anti-thrombin III isolated from the culture media of *Saccharomyces cerevisiae* BY4741 was biologically active, as could be shown by progressive anti-thrombin activity and heparin cofactor activity.

Keywords: Anti-thrombin, *Saccharomyces cerevisiae*, pYES2/CT vector, Ni²⁺-NTA chromatography, His-tag, BY4741.

INTRODUCTION

Anti-thrombin III (ATIII) plays a critical role in maintaining the flow of blood. Blood coagulation is mediated by a series of serine proteases. Anti-thrombin III is a potent inhibitor of Factors IXa, Xa [1], XI [2] and XIIa [3]. ATIII is synthesized in the liver and has a plasma level of approximately 150mg/L with molecular weight of 58KDa [4]. The primary amino acid structure of human ATIII had been completely determined by Petersen et al and it was reported that, the protein has approximately 430 amino acid residues, 4 glucosamine-based oligosaccharide side units, and 3 disulfide bridges [5]. Serine protease inhibitors (serpins) constitute a family of homologous proteins [6]. The similarity between the members of this group was first reported for alpha-AT, AT and ovalbumin [7]. Transient Ischemic Attack, Thrombosis, Thrombocytosis and Cerebrovascular diseases are the leading causes of death and disability around the globe. Blood clot dissolving agents which are used to dissolve the thrombus, have gained lot of importance in present day research [8]. 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 infarction and stroke, some alternative thrombolytics are still in research phase [9]. In case of booster doses of clot-busting drugs, immune responses are predictable, which lead to anaphylaxis and reduced efficacy for clot lysis, hence researchers looked into other alternatives. Recombinant proteins have been expressed in *E. coli* strains [10] which are far better characterized than any other microorganisms. ATIII has been expressed by rDNA technology using different expression systems like *E. coli*, Chinese hamster ovary cells and *Pichia pastoris*, but yeast derived recombinant ATIII (rATIII) has been reported only by Broker et al. [11]. In this work, ATIII was cloned and expressed in *Saccharomyces cerevisiae* BY4741. The present work employs various yeast strains, hosting compatible expression vectors, which are capable of selection and replication in both *E. coli* and yeast, particularly *Saccharomyces cerevisiae* BY4741 [12].

MATERIALS AND METHODS

Strains and reagents

pGEM-T easy vector with ATIII construct for cloning and pYES2/CT vector for expression were procured from Genewiz, USA. The *S. cerevisiae* BY4741 strain used for anti-thrombin expression was obtained from ATCC 4040002, USA. The *Escherichia coli* strains used were TOP10 and DH5a from Invitrogen. The enzymes used to

manipulate RNA or DNA were obtained from New England Biolabs (Beverly, UK), unless otherwise stated.

Plasmid and Cloning

pGEM-T easy vector along with ATIII cDNA construct was used, allowing easy exchange of vector components by restriction digestion and inserting sequences [13]. cDNA clone incorporated in pGEM-T easy vector was procured from Genewiz, USA on Whatman FTA elute card (Cat. No.: B16217-1/K49639) and plasmid was prepared as described by the manufacturer. A full-length ATIII cDNA was amplified by PCR utilizing P1/P2 as a primer pair in which pGEM-T gene construct served as a template. The pGEM-T cDNA gene was digested via restriction enzymes, EcoRI and XhoI (NEB, R0101S & R0146S) that would allow cloning into the pYES2/CT expression vector. The 1425bp ATIII cDNA was subcloned into a yeast vector, pYES2/CT, digested with EcoRI & XhoI (Figure 3). The proper orientation of the cDNA insert was confirmed by restriction enzyme analysis.

Primer sequences

Primers were designed using NCBI/Primer-BLAST and oligonucleotides were synthesized by Bioserve, India (100 pMol/μL). The following primers (P1/P2) 5'-GAATTCATGTATTCCAATGT-3' for sense strand, with EcoRI site at 5' end and 5'-ATCATCACCCTAACTCGAG-3' for reverse strand with XhoI at 3' end were used in standard polymerase chain reactions (PCR) to verify the presence of the ATIII gene in the recombinant *Saccharomyces* DNA (underlined sections of the primer sequence indicates restriction sites).

Transformation

S. cerevisiae competent cells were obtained (Life Technologies, Hungary) and transformed by using *S. cerevisiae* EasyComp Transformation kit as per manufacturer's instruction; 0.5μg of pYES2/CT vector, with a His-tagged ATIII insert was added to 100μl of *S. cerevisiae* competent cells [14], mixed gently and placed on ice for 30 minutes. Competent cells were heat shocked for 10 minutes at 42°C and immediately transferred to ice for 2 minutes. 900μl of YPD Broth with 2% glucose, 2% tryptone and 1% yeast extract was added, and tubes were incubated for one hour at 30°C shaking. Transformed cells were plated onto YNB-URA def agar plates containing 50μg/ml ampicillin. A negative control plate was prepared by plating out untransformed yeast cells onto YNB -URA

def agar plates containing 50µg/ml ampicillin. The next day colonies were selected and inoculated into 10ml of YNB-URA def broth (HiMedia) containing 0.67 % Yeast nitrogen base, 0.192% complete supplement mixture without uracil, 2% dextrose, 50µg/ml ampicillin and allowed to grow overnight. The following day, 2.5ml of the culture was inoculated into 500ml flasks in YNB-URA def broth containing 50µg/ml of ampicillin. Cultures were centrifuged at 8,000rpm in a MicroCL 21 microcentrifuge (Thermo) for 20 minutes. Supernatants were resuspended in 5ml of Protein Extraction Reagent (B-PER, Thermo) per gram of wet cell paste followed by the addition of 1µl/ml Benzonase (Millipore). This was separated into 50ml conical tubes, placed on a rotary shaker for 20 minutes and centrifuged at 11,500rpm in a MicroCL 21 microcentrifuge (Thermo) for 20 minutes.

Protein Purification

Anti-thrombin was purified from the cleared lysate using Ni²⁺-NTA His-Bind Resin. 1ml of Ni²⁺-NTA His-Bind Resin (Qiagen), per purification, was washed with 4ml of 1X Ni²⁺-NTA Bind Buffer (300mM NaCl, 50mM sodium phosphate buffer, 10mM imidazole, pH 8.0), the beads were allowed to settle by gravity or centrifuged at 1000rpm for 5 minutes, and the top bind buffer layer was removed. 4ml of cleared lysate was loaded onto the prepared Ni²⁺-NTA His-Bind slurry and mixed gently by shaking at 4°C for at least 60 minutes. This was then loaded onto a disposable polypropylene column (1-5ml bed volume; Pierce Biotechnology), flow through was collected (aliquot taken for SDS-PAGE analysis) and 3 washes were carried out using Ni²⁺-NTA Wash Buffer (300mM NaCl, 50mM sodium phosphate buffer, 20mM imidazole, pH 8.0), (aliquots of washes taken for SDS-PAGE analysis). 4x 500µl elutions were performed with Ni²⁺-NTA Elution Buffer (300mM NaCl, 250mM imidazole, 50mM sodium phosphate buffer, pH 8.0) and were collected in 4x 1.5ml eppendorf tubes. Elutions were dialysed against 1L cold 1X PBS overnight (dialysis tubing size - 14.3mm, molecular weight cut off 12-14 KDa). Free imidazole is removed from the sample by dialysis at 4°C against a buffer of choice.

SDS-PAGE

Protein concentrations were determined using Bio-Rad protein assay (Bio-Rad) using bovine serum albumin as a standard. SDS-PAGE was performed on 12% polyacrylamide gel and 4% stacking gel under reducing conditions. All samples (Flow through, Washes and elutions) were prepared by boiling them at 100°C for 5 min in 0.125 M Tris-HCl buffer, containing 4% SDS (w/v), 20% glycerol (v/v), 0.1% bromophenol blue, and 10% 2-mercaptoethanol (v/v). 15µl of sample was loaded per lane. Bio-Rad molecular weight markers were used according to the manufacturers' instructions. The protein bands were stained with GelCode Blue (Pierce Chemical).

Western blot

For visualization of intracellular as well as extracellular specific proteins, Western blot analysis was performed. After gel electrophoresis, transfer buffer was prepared (per liter: 50 mL NuPAGE 20 x Transfer Buffer, 100 mL MeOH, 1 mL Antioxidant for reduced samples; 250 mL per blotting chamber), in which sponges and filter paper (Whatman® 3mm Chr) were soaked. The nitrocellulose membrane (BioRad Trans-Blot pure nitrocellulose membrane 0.2 µm) was applied on the gel, according to the manufacturer's instructions and the blotting chamber was filled with Transfer Buffer. Blotting was mediated at 50 V for one hour. Thereafter, the membrane was blocked with 25 mL blocking solution (1 x PBS, 0.1 % [v/v] Tween 20, 1% [w/v] skim milk) for one hour and washed three times for 10 min with PBST, which was followed by incubation with Anti-Anti-thrombin antibody (Abcam, ab126598; 1:1000 dilutions, as instructed) in 7 to 10 mL blocking solution for one hour or overnight. After washing three times for 10 min with PBST, it was incubated with Goat Anti-rabbit antibody-HRP (Abcam, ab6721; 1:5000 dilutions, as instructed).

RESULTS AND DISCUSSION

Plasmid and cloning

pGEM-T was isolated from *TOP10* and 10µl of the purified plasmid was loaded on 1% agarose gel along with marker. In lane M,

Molecular marker procured from Fermentas was used for the determination of plasmid weight. While in Lane 2 and 3 the purified plasmid from *TOP10 E. coli* strain was loaded to check the purity. The molecular weight of plasmid was about 4.5kb; we obtained relatively pure plasmid that gave a considerable yield (Figure 1).

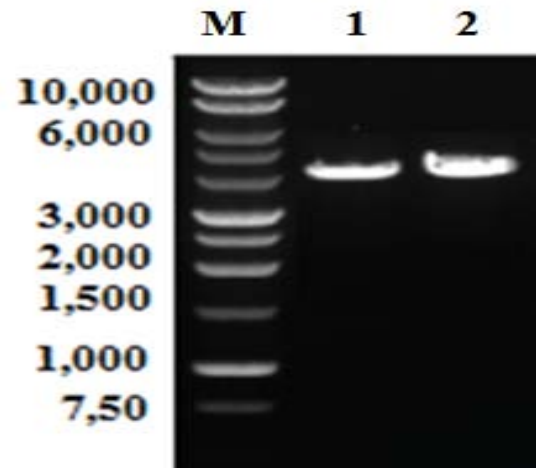


Fig. 1: Plasmid DNA was isolated from overnight *TOP10* culture and analyzed by 1% agarose gel electrophoresis. Lane M shows marker, lanes 2 & 3 show purified plasmid from *TOP10 E. coli* strain

PCR amplification of the cDNA was carried out using specific forward and reverse primers (Figure 2). The amplified cDNA of size 1425bp was then inserted into pYES2/CT vector using restriction enzymes, EcoRI and XhoI. 10µl of each 50µl reaction were electrophoresed on a 1.2% agarose gel stained with ethidium bromide. On Lane 1, size markers (1kb ladder, Fermentas) was loaded and remaining lanes show PCR products ranging to 1425bp obtained using P1/P2 set of primers.

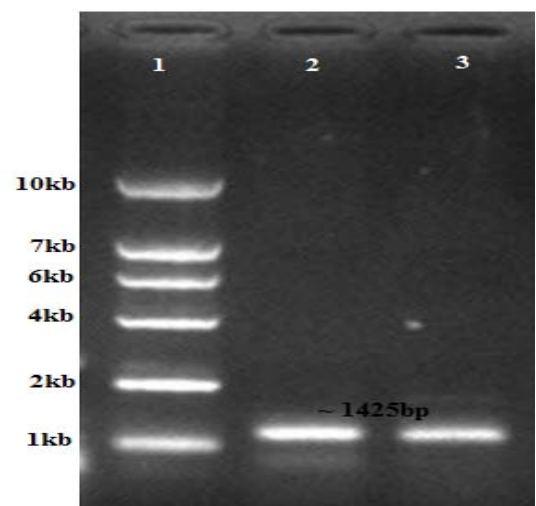


Fig. 2: Samples of the PCR products were analysed on a 1.2% agarose gel and stained with ethidium bromide. Lane 1 shows size markers of a DNA ladder, lanes 2&3 show the position of migration of the anti-thrombin product (predicted size, 1425 bp).

Digestion of pYES2/CT with restriction enzymes

We have used EcoRI and XhoI restriction enzymes to digest the DNA successfully (Figure 3). Typically, analytical digests were carried out in a total volume of 20 µl containing 5µg of plasmid DNA. Digests for

subcloning applications were typically carried out in a 50µl volume containing 25µl plasmid DNA. 5µl aliquots of the reaction mixture were taken every hour until complete digestion could be visualised after agarose gel electrophoresis. 10µl of undigest DNA and double digest was loaded to check the digested pattern along with DNA marker to confirm its molecular weight.

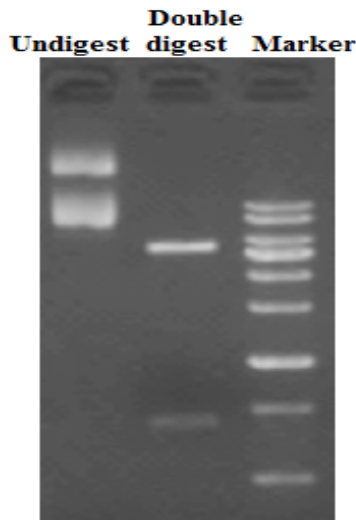


Fig. 3: Restriction analysis of pYES2: Lane 1 shows Uncut DNA/Undigested DNA (pYES2); Lane 2 shows Digests of EcoRI & XhoI; Lane M: Marker (Broad range)

Protein Purification and Analysis

Anti-thrombin was purified from culture (grown and induced in YNB-URA def medium) supernatant by affinity chromatography using a Nickel-Column. Figure 4 shows the SDS-PAGE analysis of the load, wash and eluted fractions using antibodies. However, as shown in lane FT of Figure 4, similar proteins also detected in the fraction that were the products of host system, these bands which were not detected by antibodies in western blot. The gel showing the wash fraction (lane W1, W2&W3) also contained few similar bands. Both the eluted fractions (lane E1&E2) showed a single band with a similar mobility as standard which was procured from NIBS, UK.

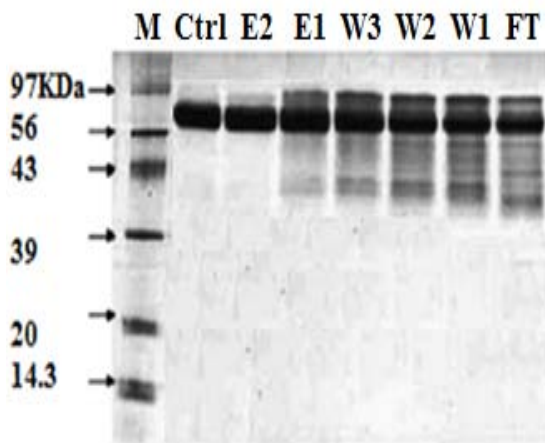


Fig. 4: Analysis of recombinant anti-thrombin using SDS-PAGE. His-tagged recombinant anti-thrombin was extracted from *Saccharomyces cerevisiae* culture media through binding to Ni²⁺-NTA His bind resin. Aliquots of flow through (FT), washes (W1-3)

and elutions (E1&2) of anti-thrombin along with medium range molecular weight marker were analyzed using 12% SDS-PAGE.

Western blot

In order to assess the immunological relationship between purified recombinant anti thrombin, the material eluted from the affinity column was submitted to SDS-PAGE and blotted onto nitrocellulose membranes. The 58kDa protein band strongly reacted with this antibody, indicating that the purified protein is anti-thrombin. A representative Western blot showing the reactivity of purified anti-thrombin (from two different extracts) with the Anti-Anti Thrombin antibody (Abcam, ab126598) at 1/1000 dilution and Goat Anti-rabbit antibody-HRP (Abcam, ab6721) at 1/5000 dilution is shown in Figure 5.

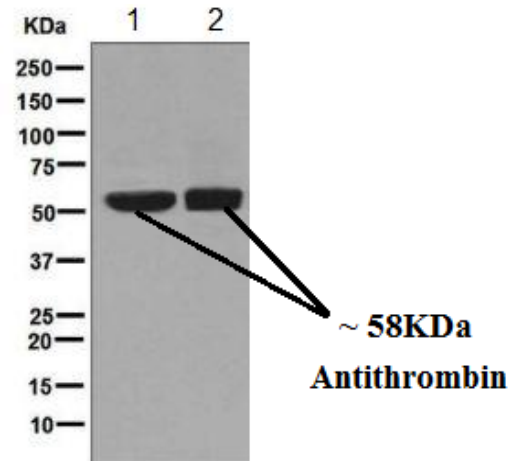


Fig. 5: Western blot of recombinant anti-thrombin from two separate purification runs. Approximately 20 µg of purified protein was loaded onto 12% SDS-PAGE and transferred to nitrocellulose membranes and Western blot was carried out using ab126598 antibody against anti-thrombin.

CONCLUSION

Apart from the inhibition of thrombin and other activated clotting factors, anti-thrombin may also possess the ability to modulate the cellular expression of pro inflammatory cytokines and pro coagulant tissue factor, contributing to the rationale for its use in the treatment of sepsis. Now a days thrombolytic therapy requires an ideal drug against to thrombosis. The recombinant bacterial protein produced by r-DNA technologies has more scope in the healing of thrombin related diseases. As developments in molecular cloning, recombinant anti-thrombin has been produced in soluble form in order to minimize drawback of native anti-thrombin from industrially feasible vector pYES2/CT. In this study, we selected human anti-thrombin - one of the important anticoagulant proteins in human plasma proteins and expressed recombinant human anti-thrombin by construction of yeast vector pYES2/CT containing human anti-thrombin cDNA sequence shown the anti-thrombin activity. The purified protein having the molecular weight of 58 KDa on 12 % SDS-PAGE was further characterized with western blotting. Still lot of research has to be done to minimize the risks during thrombolytic therapy. In future it will be ideal drug for the patients who need thrombolytic therapy.

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