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Application of pET28a Vector for the Overexpression of Recombinant Human Tim23 Protein in *E. coli* and Its Validation by Immunoblotting

Chandra Shekhar Anugula[1], Karuna Rupula[2,*]

Abstract

This study aimed to produce recombinant human Tim23 protein by molecular cloning, expression, and purification. Amplification of human TIM23 was carried out by polymerase chain reaction (PCR) and cloned into DH5α cells. The pET28a-human TIM23 was transformed into BL21DE3 and expressed the gene by IPTG induction. Recombinant pure human Tim23 antigen was prepared, and injected into rabbits and polyclonal antibodies were raised, which was confirmed by western blotting. The transformed DH5α colonies screened for recombinant TIM23 on agarose gel electrophoresis depicted a specific band corresponding to ~5.4kb pET28a vector and a band at ~0.63 kb confirmed the clone. Overexpression of recombinant human Tim23 was confirmed by SDS-PAGE in BL21DE3 bacterial cells followed by purification by affinity chromatography. Furthermore, polyclonal antibodies were raised and validated by immunoblotting. This study concludes a fundamental and comprehensive study on the molecular cloning, expression, purification, and raising of antibodies against recombinant human Tim23. Characterization of the Tim23 protein will further enhance the existing knowledge on the biogenesis of mitochondria. The polyclonal antibody of Tim23 could be a useful tool for validating regulatory mechanisms in mitochondria.

Keywords: Mitochondria, recombinant protein, Tim23, cloning, polyclonal antibodies

INTRODUCTION

Mitochondria are semi-autonomous cellular organelles that are found only in eukaryotic organisms. The mitochondrial number is dynamic and varies from cell to cell, depending on the energy requirement of the cell. The chemical energy, adenosine triphosphate (ATP), is synthesized in the mitochondria of almost all cells. Mitochondrial organelle biogenesis requires the synthesis of several proteins. These include mitochondrial DNA-encoded proteins as well as nuclear-encoded proteins that respond to environmental conditions and the physical state of cells. Mitochondrial proteins encoded by the nuclear genome are targeted to the mitochondria by the presence of the signals in them. Very few proteins are destined for transport to the mitochondria, even without signal sequences. The transport of proteins to mitochondria is facilitated by mitochondrial complexes referred to as translocase of the outer membrane (TOM) and translocase of the inner membrane (TIM). Thus, it is very important to understand and elucidate the basic mechanisms of these complex (TOM and TIM) proteins in detail. Much of the earlier studies to understand the basic mechanism behind mitochondrial biogenesis have been carried out in

***Author for Correspondence**
Karuna Rupula
E-mail: karunarupula@osmania.ac.in

[1]Research Scholar Department of Biochemistry University College of Science Osmania University, Hyderabad Telangana India
[2]Associate Professor and Head, Department Biochemistry, University College of Science, Osmania University, Hyderabad, Telangana, India

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lower eukaryotic organisms (i.e., yeast) and cell-free systems [1]. Later, many research groups worked on the different transport systems present in mitochondria and five import machinery have been identified to date, including (i) TOM channel- main entry gate for all pre-sequence proteins [2], (ii) TIM22 and 23 complexes [3], (iii) SAM (sorting and assembly) machinery [4], (iv) mitochondrial import and assembly (MIA) machinery [5], and (v) MIM (mitochondrial import) machinery [6].

Since their identification, various studies have been carried out by several research groups on TOM and TIM complexes and their importance in mitochondrial biogenesis [7]. Recently, proteomic approaches such as mePROD^{mt} (protein uptake assay) have been used to detect newly synthesized mitochondrial proteins, and a pulsed-SILAC labeling experiment showed the effect of inhibitors on protein import machinery, protein import rates, and protein uptake under stress conditions [8].

Protein channels with a large number of interacting partners have yet to be identified for a few mitochondrial proteins that are translated into the cytosol. Although the mitochondrial complexity of the import machinery is well identified and understood in lower organisms, detailed studies are needed to determine the assembly of channel proteins and find new pathways for sorting proteins in mammalian cells, especially in human mitochondria.

From these studies, the basic import complex channel proteins must be characterized thoroughly to identify the possible pathways. Further specific investigations on the TOM and TIM complexes of mitochondria in higher eukaryotes, especially in humans, have not been reported so far. It is cumbersome to investigate the mitochondrial complexes together. Therefore, candidate partners of complex proteins must be studied separately. With the advancement of genetic engineering and cloning techniques in the past few decades, a large number of studies have been carried out with the aid of recombinant proteins under *in vitro* conditions. Recombinant proteins are widely used in different platforms, such as pharmaceutical, human, and animal health, agriculture, protein-protein interactions, cell biology modulation, inhibition assays, affinity/specificity, and antibody production. Furthermore, biochemical characterization and antibody production against proteins under *in vitro* conditions require large quantities of pure fractions.

Hence, the present study involved the molecular cloning, expression, and purification of recombinant human *Tim23*.

MATERIALS AND METHODS

Chemicals and Reagents

Acrylamide, agarose, sodium dodecyl sulfate (SDS), tetramethyl ethylene diamine (TEMED), tween-20, PMSF, imidazole, ethylene diamine tetra acetic acid (EDTA), lysozyme, ammonium persulfate (APS), and phenyl methane sulphonyl fluoride (PMSF) were procured from Sigma-Aldrich (St. Louis, MO, USA). The DNA ladder, dNTP, and protein markers were purchased from Fermentas (Massachusetts, USA). Agar powder, chloramphenicol, ethidium bromide, and kanamycin were procured from HiMedia (Mumbai, India). All other reagents and chemicals used were of analytical grade and freshly prepared for the present study.

Media and Antibiotic

The present study was carried out using a standard Luria Bertani (LB) medium composed of yeast extract (1%), tryptone (2%), and NaCl (2%). The culture media were used as liquid broth and solidified by adding agar. Further, antibiotic (marker) Kanamycin was used as an antibiotic marker.

Enzymes

The cloning of human *TIM23* was carried out using enzymes T4 DNA ligase, Pfu polymerase, and restriction endonucleases (*EcoRI* and *XhoI*), which were procured from Fermentas.

Vector

The pET28a is an *E. coli* expression vector (~5.4kb in size) containing a strong T7 RNA polymerase promoter that was used in the present study which has a 6X histidine tag and kanamycin antibiotic resistance marker gene.

Primers

TIM23 was amplified using the forward primer 5′-CCCAGAATTC ACCATGGGAAGGAGGCGGGGGAAGCGGCAACAAA-3′ and reverse primer 5′-CCCACTCGAGTCAAGAGTGACTGTTGGAGCAAGGAGCCTTT-3′.

Agarose Gel Electrophoresis

The TAE buffer (50X) was prepared by adding Tris base (242 g), sodium EDTA (32.2 g), and glacial acetic acid (57 mL). The final volume was made up to 1 liter with double double-distilled water.

Reagents and Buffers Used for Gel Extraction

Solubilization and binding buffer, wash, and elution buffer (10 mM Tris-HCl, pH 8.5) were used for the extraction of DNA from gels.

Buffers and Solutions for SDS-PAGE

Tank buffer (10X): To 800 mL of distilled water, 144.4 grams of glycine, 30.3 grams of Tris base, and 10 g of SDS were added, mixed, and diluted to 1000 mL with distilled water. One liter of 1X tank buffer was prepared by mixing 100 mL of 10X buffer and 900 mL of distilled water. Buffer for resolving gel (1.5M Tris-HCl, pH 8.8) and stacking gel (1.0M Tris-HCl, pH 6.8). Acrylamide (30%) was prepared by adding acrylamide (29.22 g) and bisacrylamide (0.78 g) to double-distilled water (100 mL) and stored at 4°C. SDS sample buffer (4X) prepared by mixing 2.4mL of 1M Tris-HCl (pH 6.8), 4 mL of glycerol, 0.8 g of SDS, 4 mg of bromophenol blue, 0.5 mL of β-mercaptoethanol, and made up to 10 mL with distilled water. The staining solution was prepared by mixing 250 mg Coomassie brilliant blue (R-250) in ethanol (40%) and acetic acid (10%). The destaining solution was prepared by adding 10 mL of ethanol and 7.5 mL of glacial acetic acid and made up to 100 mL with distilled water. A gel electrophoresis unit (Bio-Rad), blotting apparatus, centrifuge (Thermo Scientific, USA), and polymerase chain reaction (PCR) machine (Eppendorf, Germany) were used for this present study.

Methodology

Polymerase Chain Reaction

The first step in cDNA preparation was using a reverse transcription kit (Bangalore Genie, India), and RNA was extracted from the HeLa cell lines. cDNA (~500ng) prepared was used as a template to amplify the human *TIM23* gene (ORF) using the following specific primers by PCR. The *TIM23* gene was 630 bp in length. Gene-specific primers were designed and used for human *TIM23* ORF. The *TIM23* gene was amplified by PCR using 0.5 µM of forward and reverse primers, 50 µM of each of the four dNTP, and DNA polymerase (0.5 U of DNA polymerase. The reaction was run by maintaining the lid temperature at 104°C. The number of cycles was 32, which included three steps: denaturation (94°C for 4 min), annealing (55°C for 1 min), and extension (72°C for 1:30 Min). Amplicons were resolved by agarose gel electrophoresis (1%).

Agarose Gel Electrophoresis

Preparation of agarose gel (1%): Agarose (0.5 g) was weighed, added to 50 mL of 1X TE buffer, and 3 µL of ethidium bromide (EtBr) was added. The gel was then boiled and cooled to solidify. The samples were loaded into the wells after adding 6X gel loading of sample dye. An electrophoretic run was performed at 90V until the sample dye reached the other end of the gel. The separated DNA fragments were examined using a UV-light transilluminator.

Isolation of pET28a Plasmid by Miniprep (QIAprep) Kit

All reagents and buffers provided in the kit (QIAGEN) were used for plasmid isolation. DNA was eluted in 30µL of elution buffer, and the concentration of DNA was found to be ~600 ng μL^{-1}. Purified plasmid DNA was subjected to restriction digestion.

Restriction Digestion (Double) of TIM23 (Insert) and pET28a (Vector)

One unit each of *EcoRI* and *XhoI* restriction enzymes (1 U each) was added to vector DNA and inserted, followed by 1 µL of restriction digestion (10X) buffer, and finally, the volume was made up to 50µL with sterile Milli-Q water (18.2 MΩ.cm). The reaction mixture was incubated at 37°C for 3 h in a digital water bath, and the digested products were separated by agarose gel (1%) electrophoresis.

Ligation

Plasmid DNA (~150 ng μL^{-1}) and insert DNA were added in a 1:3 ratio. One unit of T4 DNA ligase enzyme (1 µL) was added, and the volume was made up to 10 µL using sterile Milli-Q water. The reaction mixture was then incubated overnight at 22°C in a digital water bath.

Transformation of Ligation Product into E. coli Bacterial Cells (pET28a-TIM23)

Prepared $CaCl_2$-treated competent cells (DH5α and BL21DE3. Plasmid DNA (ligated product pET28a with *TIM23*) was added to competent cells and incubated for 30 min. Following a 90-second heat shock treatment at 42°C, the cells were immediately placed on ice and left to stand for 3 min. The cells were then transferred to 1 mL of LB broth and incubated at 37°C for 1 h. Centrifugation was performed at 6000 rpm for 5 min. The transformed cells were resuspended in LB broth and transformed onto solidified LB plates with an antibiotic (kanamycin; 25µg mL^{-1}). The plates were then incubated at 37°C overnight.

Restriction (Double) Digestion of pET28a-TIM23 and Transformation into Bacterial Cells

Individual pET28a plasmids containing the *TIM23* insert were digested with restriction enzymes (*EcoR I* and *Xho I*) at 37°C for 3 h, and the digested products were separated and observed by performing 1% agarose gel electrophoresis. Later, sequences were confirmed using an auto sequencer (Vimta Labs Hyderabad). Subsequently, a general transformation was carried out, as mentioned earlier.

Overexpression of Recombinant Human Tim23 Protein

Overnight cultures of *E. coli* (BL21DE3) cells expressing Tim23 protein were inoculated into 1 liter of liquid broth medium containing kanamycin (25 µg mL^{-1}) and incubated at 37°C in a platform shaker at 150-200 rpm for 3 h until the cultures reached OD (0.4, 0.8,1.0) at λ600 nm. Cells were then treated with different concentrations of IPTG (0.1, 0.5, and 1 mM) and subjected to induction at 37°C for 3 h. The culture samples were then centrifuged at 8000 rpm for 10 min at 4°C. The cell pellet was then lysed with 1/20th volume of bacterial lysis buffer (50 mM Tris-HCl pH 8.0), followed by sonication (probe method) with 7 pulses (15 s/pulse) on ice until the lysate was clarified. Samples were further clarified by centrifugation at 12000 rpm for 20 min at 4°C. The pellet and supernatant samples were analyzed using SDS-PAGE.

Polyacrylamide Gel Electrophoresis

Resolving gel (12%) of 5 mL was prepared by adding 1.6 mL of water, 1.3 mL of 1.5M Tris-HCl (pH 8.8), 2.0 mL of 30% acrylamide, 0.05 mL of 10% SDS, 0.05 mL of APS and 0.002 mL of TEMED. Stacking gel (5%) of 2 mL was prepared by adding 1.4 mL of water, 0.33 mL of 1.0 M Tris-HCl (pH 6.8), 0.25 mL of 30% acrylamide, 0.02 mL of 10% SDS, 0.02 mL of APS and 0.02 mL of TEMED. Samples were prepared by mixing with sample buffer in a 1:3 ratio. Electrophoresis was performed after loading samples in wells for 3 h at 100 V. Gels were removed from the plates and placed in a staining solution for 2 h on a rocker. The samples were gently washed with water, and the destained solution was added. Destaining continued until the bands were visualized. Images were obtained by scanning and were used for analysis.

Affinity Purification

The ethanol-stored Ni-NTA affinity beads were initially equilibrated with the washing buffer. Nickel (Ni) beads (1 mL) were centrifuged at 5000 rpm for 2 min. The supernatant was discarded. The protein samples were equilibrated in 10 M urea. Tris-HCl buffer was added and kept for end-over-end rotation for 2 h at 37°C. The sample was then loaded onto the affinity column, and the flow-through was collected and saved for further use. The affinity column was extensively washed with washing buffer (8 M urea, 50 mM Tris-HCl, pH 8.0 and 1mM imidazole) and elution buffer (8 M urea, 50 mM Tris-HCl, pH 8.0 and 0.4 M imidazole) was passed through the column and the eluted protein fractions were collected. Then, all purified protein fractions were pooled. The protein concentration was assessed using a Bradford reagent (Amersco). The purified fractions were evaluated using SDS-PAGE to confirm the affinity of purified human *Tim23*.

Polyclonal Antibodies Production

Polyclonal antibodies were raised against rHuman Tim23 in rabbits (New Zealand white rabbits, 2.5 kg weight, 2-3 months old, procured from NCLAS-NIN Hyderabad). Pre-immune serum samples were collected. Affinity-purified human Tim23 protein (~1 mg) was separated using SDS-PAGE (12%) and homogenized. It was then mixed with Freund's complete adjuvant (Bangalore GeNei, India) and injected subcutaneously into the rabbits. After 3 weeks of interval time booster doses of the protein (~0.5 mg) were administered. Booster doses were prepared by mixing Tim23 protein with Freund's incomplete adjuvant and injected. Two weeks after the 2nd booster dose, blood samples were collected, serum was separated, and polyclonal antibodies were evaluated using immunoblotting. A larger volume of blood (~30mL) was collected, and the serum was separated.

Western Blotting

The proteins were separated by SDS-PAGE and then transferred to a nitrocellulose membrane using a mini transfer pack (Bio-Rad) at 30 V overnight. The membrane was blocked by incubation for 1 h at room temperature with a solution consisting of TBST (Tris-HCl (20 mM), pH 7.5, NaCl (150 mM), and 0.05% Tween20) that included milk powder (5%). The blot was incubated with primary antibody (anti-human Tim23) and horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 2 h. Then, the blot was developed by using ECL (enhanced chemiluminescence) reagents (GE Health care, USA) on versa doc (BIO-RAD gel doc system).

RESULTS AND DISCUSSION

Mitochondria are referred to as powerhouses of cells, as they generate most of the energy through the OXPHOS system by oxidation of macromolecules, including carbohydrates, proteins, and lipids. The OXPHOS complex and its status play key roles in cellular functions and are potential therapeutic targets [9]. When cells are under stress or in response to environmental cues, a variety of signals, including ATP depletion, trigger mitochondrial biogenesis. Mitochondrial biogenesis is an important biological process by which new mitochondria are created in a cell. Cellular metabolism is also greatly influenced by mitochondria in the creation and destruction of free radicals [10]. It is believed that a cell is protected by a higher mitochondrial copy number or mass.

There are various functions of mitochondria apart from the generation of ATP, including its role in apoptosis, cancer, and aging. Dysregulation of mitochondrial proteins due to mutations has been shown to cause various neurological disorders such as Parkinson's disease and Alzheimer's disease [11]. In the last few decades, much research on mitochondria has been carried out in eukaryotic model organisms such as budding yeast (*Saccharomyces cerevisiae*). Mass spectrometry-based proteomics has significantly shaped our understanding of the *S. cerevisiae* proteome of mitochondria [12], its sub-compartment proteins, and the regulatory mechanisms that control mitochondrial homeostasis [13].

Palmfeldt and Bross's group (2017) employed mass spectrometric methods and bioinformatics to gain a detailed understanding of mt protein topography functions and illustrated the importance of proteomic analysis in a variety of illnesses, ranging from hereditary problems to complicated illnesses

such as cancer, type 2 diabetes, and neurodegenerative diseases [14]. A study with recombinant proteins of any cell organelle is an important aspect in terms of biogenesis, as it involves making pure products in required quantities. Recently, Revathi et al. (2021), who studied recombinant mammalian mitochondrial ribosomal small subunit (MRPS) protein expression, affinity purification, and protein-protein interactions, suggested their potential role in tumorigenic cellular processes [15].

According to previous studies, a recombinant protein in a bacterial system does not undergo any post-translational modification [16]. Hence, in the present study, the best and most well-established cloning and expression platform, *Escherichia coli* (BL21DE3), was used for the production of recombinant human *Tim23*. *E. coli* cells are ideal for such studies because of their fast growth (doubling time of 20 min) and the procedure is cost-effective [17].

In the present study, we focused on the preparation of pure recombinant human Tim23 (full-length) by amplification of the human *TIM23* gene, cloning it into a bacterial vector overexpressing the recombinant protein, and purification. Following this, polyclonal antibodies against recombinant human Tim23 in rabbits were also produced.

Amplification of Human *TIM23*

Human cDNA was prepared from total RNA extracted from HeLa cell lines by reverse transcription. The cDNA was then used as a template for the amplification of the human *TIM23* gene (ORF) by PCR. Specific forward and reverse primers were used to amplify the human *TIM23*. The PCR-amplified products were analyzed by agarose gel electrophoresis using EtBr staining for visualization of the gel bands. A single discrete band was obtained, as shown in the gel electrophoretic image in Figure 1. The amplified product of the *TIM23* gene corresponded to (~0.63kb) molecular weight in comparison with the molecular weight standards used.

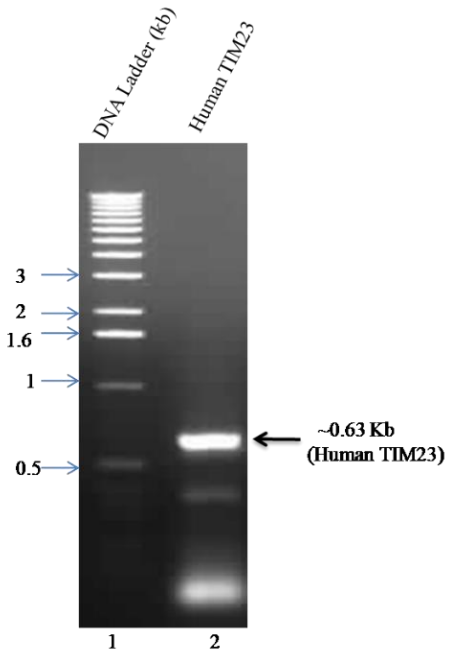

Figure 1. PCR analysis of full-length human *TIM23* by agarose gel electrophoresis. 1 kb DNA ladder loaded on Lane 1 and PCR-amplified sample loaded on Lane 2. The arrow indicates the amplified human *TIM23* gene (~0.63 kb).

Restriction Digestion of Insert and Vector

Double digestion of pET28a and *TIM23* was carried out with *EcoR I* and *Xho I*. Digested products were separated by agarose gel (1%) electrophoresis, which exhibited a single dense band, as shown in

Figures 2 and 3. The single band of double-digested human *TIM23* (~0.63kb) confirmed the purity of the *TIM23* gene. Subsequently, band regions were excised and purified for ligation.

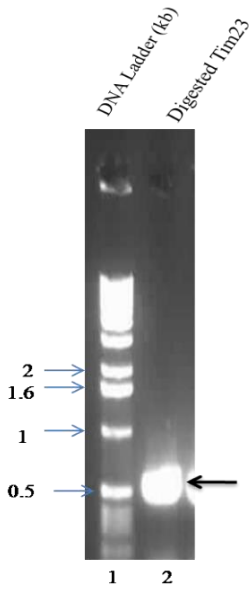

Figure 2. Separation of restriction-digested human *TIM23* by agarose gel (1%) electrophoresis. 1 kb DNA ladder loaded on Lane 1 and digested sample loaded on Lane 2.

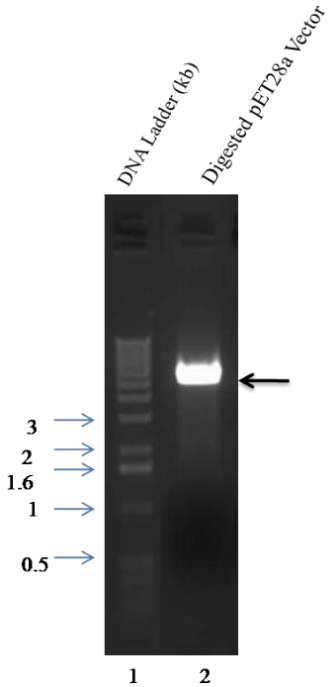

Figure 3. Separation of digested pET28a by agarose gel (1%) electrophoresis. Lane 1: 1 kb DNA ladder and Lane 2: digested sample. Double-digested bacterial vector pET28a single thick band obtained as indicated by an arrow mark.

The digested vector size was ~5.4kb confirmed by standard molecular marker DNA bands. The band region containing vector DNA was excised from the gel, solubilized in gel extraction buffer, and purified using digested vector DNA. The concentration of the vector DNA, as estimated by the nano-drop method, was found to be ~600 ng μL^{-1}.

Transformation of (pET28a-humanTIM23) into Bacterial Cells

The vector (pET28a) and *TIM23* genes were subjected to ligation using T4 DNA ligase. The DH5α competent cells were prepared and transformed into DH5α competent cells. Clear colonies were observed on agar plates (Kan^+) (Figure 4). Subsequently, the colonies were analyzed for positive clones.

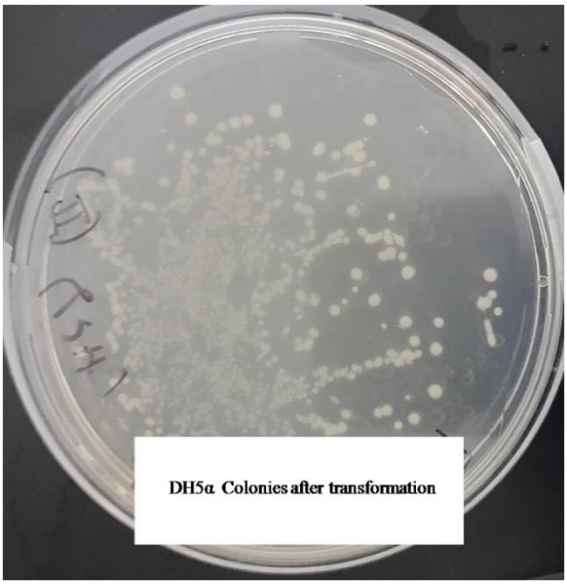

Figure 4. Bacterial colonies (DH5α) on an agar plate (Kan^+).

Screening of Bacterial Colonies

Single colonies (DH5α) were picked and transferred to an LB medium containing antibiotics (Kan^+) and incubated at 37°C overnight. Samples were treated with restriction enzymes (*EcoR I* and *Xho I*) after isolating the plasmid DNA from the cells. These were examined by agarose gel electrophoresis (1%) with ethidium bromide (EtBr) staining, as shown in Figure 5. The gels were then exposed to a UV transilluminator. The specific band corresponding to ~5.4kb confirmed the pET28a vector and a band at ~0.63 kb confirmed the insertion of DNA (*TIM23* gene) in the transformed DH5α cell extracts.

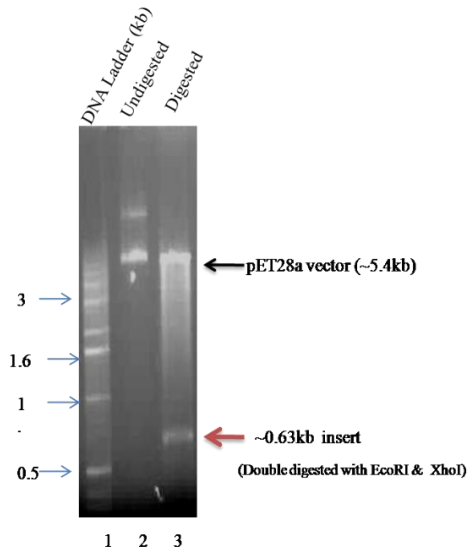

Figure 5. Restriction digestion analysis of pET28a-human *TIM23* by agarose gel (1%) electrophoresis. Screening of the colonies was carried out using restriction (double) digestion. Loaded digested and undigested samples on agarose wells and separated by electrophoresis. Lane 1, 1 kb DNA marker, Lane 2, undigested vector, and Lane 3, double-digested sample with insert.

Transformation of pET28a-Human *TIM23* into BL21DE3 Cells

Isolated and purified plasmids with the human *TIM23* gene (positive clone) were transformed into BL21DE3 cells. The colonies were grown on agar plates (Kan^+), as shown in Figure 6.

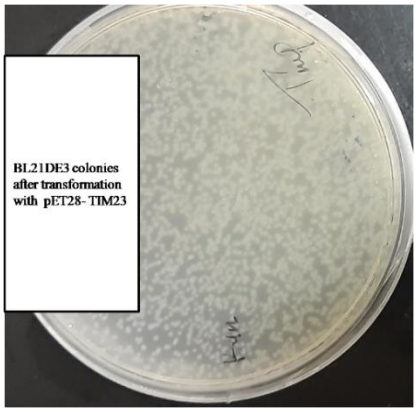

Figure 6. Bacterial colonies (BL21DE3) on an agar plate (Kan^+).

Furthermore, to carry out bioassays that reveal the mechanisms underlying mitochondrial biogenesis, it is necessary to have sufficient amounts of specific proteins. For this purpose, recombinant human Tim23 protein was overexpressed in BL21DE3 bacterial cells and purified by affinity purification.

Overexpression of Recombinant Human Tim23

Overexpression of recombinant humanTim23 was carried out as described in the methodology by IPTG. The samples were analyzed by SDS-PAGE (12%) under reducing conditions, as shown in Figure 7. All induced cells exhibited a clear thick band (~23 kDa), whereas uninduced cells did not show any thick bands at this position. This indicated the overexpression of recombinant human Tim23 in the BL21DE3 bacterial cells. These samples were subjected to sonication to isolate the pellet and supernatant fractions.

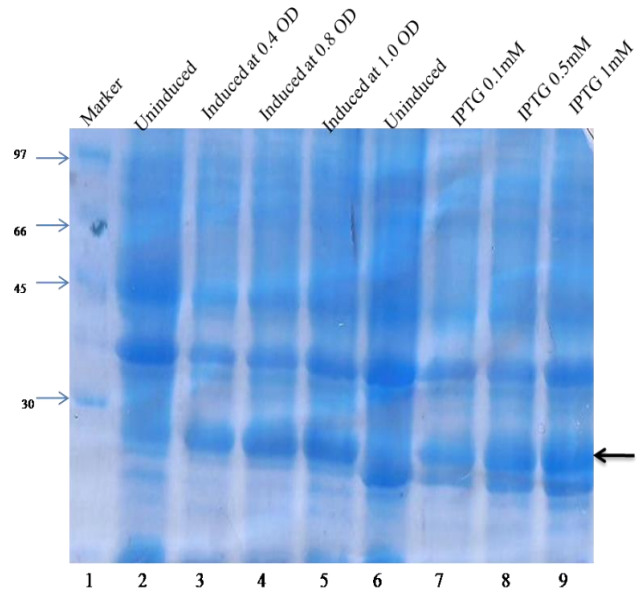

Figure 7. Expression profile of rHuman Tim23 (FL) analyzed by SDS-PAGE. Different fractions of bacteria expressed recombinant Tim23 were shown. Lanes 1, 2, 3, 4, and 5 indicate marker, uninduced, induced 0.4 OD, induced 0.8 OD, and induced 1.0 OD of samples respectively. Lanes 6, 7, 8, and 9 indicate uninduced, induced with 0.1 mM IPTG, induced with 0.5 mM IPTG, and induced with 1.0 mM IPTG samples respectively.

Analysis of Soluble and Insoluble Fractions of rHuman Tim23

Recombinant Tim23 was overexpressed as described in the methodology for IPTG induction. These samples were analyzed using SDS-PAGE (12%), as shown in Figure 8. The gel analysis clearly showed that Tim23 protein was visible in the pellet/insoluble fraction, and no other fractions showed overexpressed protein with Coomassie staining on SDS-PAGE.

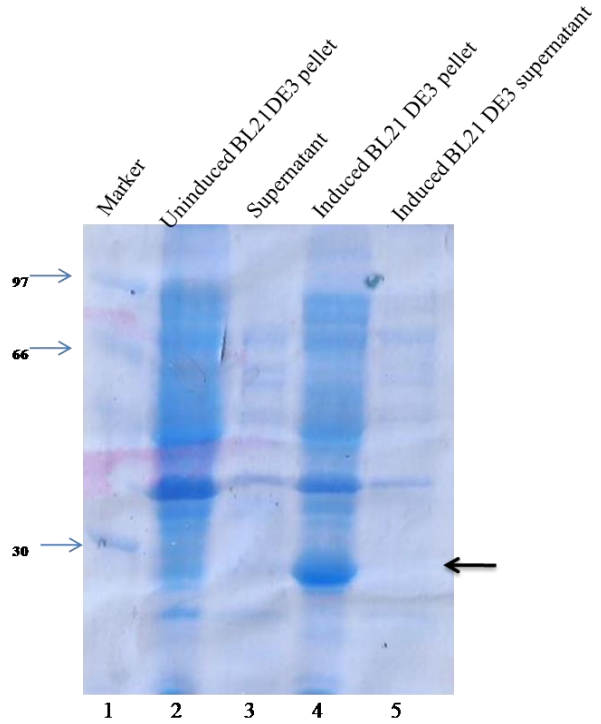

Figure 8. Analysis of pellet and supernatant fractions of full-length rHuman Tim23 by SDS-PAGE (12%). Pellet & supernatant samples of induced and uninduced cells loaded and resolved on SDS-PAGE. Induced BL21DE3 pellet Lane 4 clearly showing overexpressed of Tim23 indicated by arrow mark. Lane 1: Marker; Lane 2: Uninduced pellet; Lane 3: Uninduced supernatant; Lane 4: Induced pellet and Lane 5: Induced supernatant fraction.

Experiments using pure proteins are always superior to using crude proteins for different studies to understand multiple aspects, such as structure-function relationships, post-translational modifications, and protein-protein interactions. Hence, the purity of the protein was determined using affinity chromatography.

Affinity Purification of rHuman Tim23

Recombinant human Tim23 was purified by affinity chromatography as previously described. The purified fractions were analyzed with flow-through SDS-PAGE (12%), as shown in Figure 9. Subsequently, the fractions were pooled and used in further studies. A single discrete band of rHuman *Tim23* was observed, indicating the homogeneity and purity of the Tim23 protein isolated and purified fractions.

Another important application of recombinant proteins is the use of recombinant antigens for the production of polyclonal antibodies. Antibodies are specific and very important in the characterization, purification, and identification of their counter molecules, that is, antigens, in biological research. Many research groups have proposed the use of polyclonal antibodies using recombinant proteins in animals. Smekenov et al. (2020) raised polyclonal antibodies against a plant protein (RHT-D1A protein) in New Zealand white rabbits [18]. Accordingly, in this study, after purification, recombinant Tim23 protein was used as the antigen, and polyclonal antibodies were raised against it in experimental rabbits.

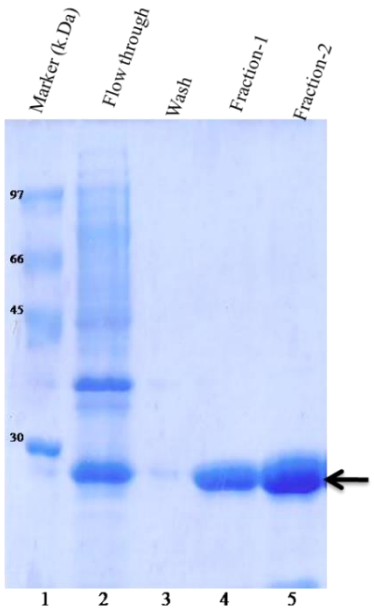

Figure 9. Analysis of purified fractions of full-length rHuman *Tim23* by SDS-PAGE (12%). The arrow mark indicates a single discrete band on gel which confirms the bacterial expressed recombinant purified *Tim23* with a corresponding molecular weight of ~23kDa as shown in Lanes 4 and 5. Lanes 1, 2, 3, 4, and 5 are indicated by a marker, flow-through fraction, wash fraction, and purified elution fraction 1 and 2, respectively.

The usual first dose of recombinant protein (antigen) administered with Freund's adjuvant in rabbits is in the range of 100–1000 µg, and for mice, it is 20–200 µg; for goats or sheep, the typical dose concentration is 250–5000 µg [19]. In the present study, polyclonal antibodies were raised against purified recombinant human Tim23 (~1 mg) protein.

Production of Polyclonal Antibody Against rHuman Tim23

Polyclonal antibodies were raised in rabbits, as explained in the methods section. Pre-immune and immune blood serum samples were prepared from rabbits and analyzed for polyclonal antibodies against human *Tim23*, as shown in Figure 10.

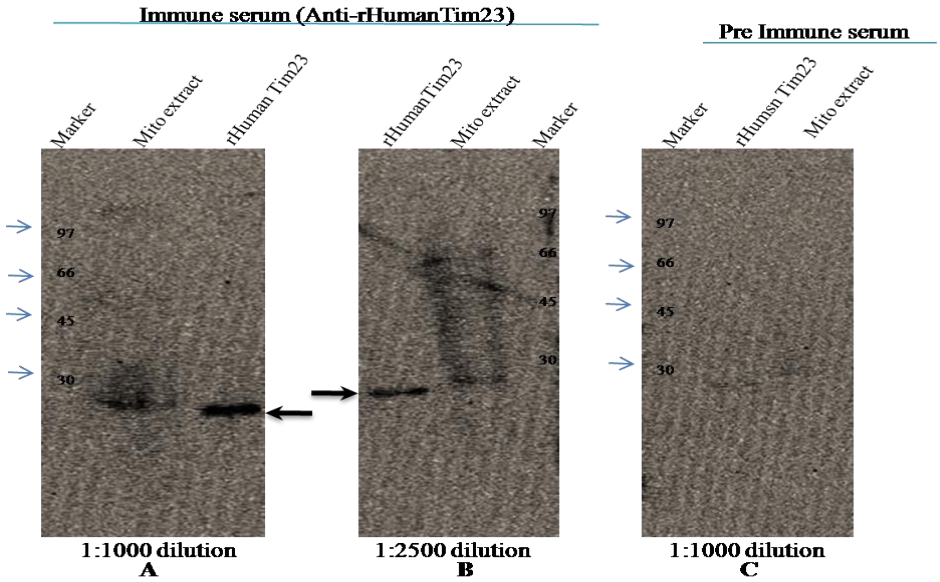

Figure 10. Screening for antibodies raised against rHuman Tim23 by western blotting.

Mitochondrial samples and purified recombinant Tim23 proteins were separated by SDS-PAGE (12%) and transferred to 0.2 μm nitrocellulose membrane as described in methodology 2.2.2.18. These blots were incubated with immune serum and pre-immune serum at dilutions of 1:1000 and 1:2500, respectively.

A single band, indicated by arrow marks, confirmed the presence of polyclonal antibodies against human Tim23 in the serum collected from immunized rabbits. No bands were present in pre-immune serum, suggesting that no pre-existing antibodies were present in rabbits against *Tim23*. These polyclonal antibodies were used for further studies on *Tim23*.

This study provides a fundamental approach for functional studies of *Tim23* and is invaluable in the field of mitochondrial biology. Various studies have strongly suggested the full potential of human mitochondrial proteomics and its applications in cell biology. Characterization of human mitochondrial proteins will further enhance the existing knowledge on mitochondrial biogenesis. Polyclonal antibodies against *Tim23* could be a useful tool for identifying regulatory mechanisms in mitochondria.

CONCLUSIONS

The present study demonstrated the successful application of molecular cloning, expression, and purification of recombinant full-length human *Tim23* in the field of mitochondrial biology. Furthermore, overexpression of *Tim23* was achieved by IPTG induction in *E. coli* with the pET28a vector and later purified by affinity chromatography. Polyclonal antibodies were raised against purified rHuman Tim23 antigen. Characterization of Tim23 further enhances the existing knowledge of mitochondrial biogenesis. Polyclonal antibodies raised against Tim23 could be a useful tool for the validation of regulatory mechanisms based on structure-function relationships, post-translational modifications, and protein-protein interactions in the mitochondria.

Author's Contribution

CSA carried out all the experimentation, acquisition, and analysis of data, and drafting of the manuscript. CSA assisted with the data analysis KR conceived, designed, and supervised the study, and revised the manuscript. All the authors have read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Ethical Approval

N/A

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