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Enhancing Protein Solubility and Functionality: The receptor of the Egg Jelly Domain of Polycystin-1 protein

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Abstract

The polycystic kidney disease (PKD-1) gene produces the polycystin-1 (PC-1) protein, which includes a substantial extracellular region housing multiple functional polypeptide motifs. In this study, one of the domains of interest is the receptor of the egg jelly (REJ). In the field of proteomics research, generating recombinant proteins in their native, soluble state and in significant quantities often presents a substantial challenge. To address these challenges in expression, we employed expression vectors that incorporate a distinct affinity fusion tag, the maltose binding protein (MBP), to facilitate the production of the REJ fusion protein in a soluble form. MBP serves as a molecular chaperone, thereby aiding in the accurate folding of the fusion protein. Our data indicate that the inclusion of the MBP tag significantly improved both the yield and solubility of the REJ protein. The results obtained from our study provide an experimental approach for the further exploration of solubility, functionality, and interactions of REJ with extracellular matrix (ECM) proteins. Moreover, this approach lays the groundwork for a systematic investigation into the impact of disease-causing mutations within the REJ module of human PC-1, with the potential to greatly enhance the overall production of fused proteins.

Keywords: Polycystin-1, Receptor of the egg jelly (REJ) domain, Phosphoramidite synthetic method

1. Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is genetically heterogeneous and inherited in humans as a dominant trait. The polycystic kidney disease-1 (PKD-1) gene is responsible for 85% of ADPKD cases (IPKDCPKD, 1995). ADPKD is genetically heterogeneous and inherited in humans as a dominant trait. The polycystic kidney disease-1 (PKD-1) gene is responsible for 85% of ADPKD cases (Cornec-Le Gall, et al., 2019). ADPKD, the most prevalent hereditary renal disease in humans, the fourth leading cause of end-stage renal disease (ESRD) among adults (Grantham et al., 2006). In ADPKD, renal function typically remains normal or near normal for several years before entering a gradual decline. Around 50% of ADPKD patients require renal replacement therapy by the age of 60 years (Chapman et al., 2012). The clinical feature of the ADPKD phenotype involves the progressive enlargement of the kidneys (Grantham et al., 2011). Which results from the development and continual expansion of multiple fluid-filled cysts originating from the tubule wall. These cysts contribute to the compression and obstruction of neighboring nephrons, causing injury to the normal parenchyma, despite the apparent maintenance of normal renal function (Luo, et al., 20023). Genetic researches enable scientists to determine the main causes behind cyst formation in ADPKD and to discover the abnormal genes that are responsible for ADPKD [6]. ADPKD is a consequence of a mutation in either the polycystic kidney disease genes PKD-1 or PKD-2, which encode polycystin-1 (PC-1) and polycystin-2 (PC-2), respectively. Since, under normal conditions, both PC-1 and PC-2 regulate the growth and development of kidney cells ((Luo, et al., 20023). ADPKD is a major health issue in the Kingdom of Saudi Arabia (KSA), and the progression of end-stage kidney disease (ESKD) is inevitable. The median age for ADPKD patients in KSA to develop ESKD is 30 years old (Alzahrani et al., 2022).

PC-1 is a transmembrane protein, the extracellular region of PC-1 has the potential to interact with ECM proteins, which are important in the signal transduction pathway, play a role in renal development, and could be a very important step in molecular cystogenesis (Collins and Wann, 2020). Evidence has shown that the disruption or loss of PC-1 activity inevitably results in subtle derangements of cellular calcium regulation, through several potential pathways, as the cellular calcium's abnormal homeostasis may subsequently alter the differentiation within affected cells (Vien et al., 2020). Furthermore, it has been reported that the receptor of the egg jelly (REJ) domain which is located in the N-terminal extracellular region of the PC-1 protein, may play a role in supporting the influx of calcium ions (Ca2+) in the PC-1 domains (Schröder et al., 2011).

Enzymes and membrane proteins exemplify protein categories that present expression challenges, yet they have gained immense significance as valuable targets with therapeutic potential. These intricately produced proteins have assumed a central role in therapeutics. Enzymes hold pivotal roles in diverse biological processes, underscoring the importance of their production in elucidating their functions and investigating their therapeutic possibilities. Conversely, membrane proteins are intrinsic to cellular membranes and participate in critical

roles such as signaling and transportation. While the hurdles linked to their expression and examination are noteworthy, their value in therapeutic contexts has fueled substantial research endeavours aimed at surmounting these challenges and realizing their potential advantages (Marjuki, Et al., 2019).

REJ domain is a crucial part of the PC-1 ectodomain, covering approximately 1000 amino acids. This module contains numerous missense mutations responsible for various diseases. Nonetheless, there is limited knowledge about the role of this region (Xu et al., 2013). Approximately 230 mutations exist, which include 80 missense mutations of the REJ region, and among these missense mutations, Kurbegovic et al predict that there are 65 are the disease-causing ones. The REJ region is encoded by PKD-1 exons15 -23 (Kurbegovic, et al., 2014). The PC-1 protein has a single domain of REJ, which is the largest domain in the N-terminal of the PC-1 protein (Gunaratne et al., 2007). REJ localized near the GPS domain at the GPSG-protein-coupled proteolytic site, PC-1 undergoes cleavage, and this process involves the entire REJ region (Babich et al., 2004). Researchers have tried to produce sufficient quantities of REJ protein to perform studies of its structure and function. While many details related to the REJ protein remain unknown, biophysical and biochemical experiments may provide further insight into its properties [15]. The REJ and GPS transmembrane domains of the protein were considered to form a channel that allowed Ca^{2+} to enter (Grieben et al., 2016).

Evaluating the expression of PC-1 as recombinant proteins presents a significant challenge due to the difficulties associated with its purification and the limited yield (Weston, et al., 2003). An alternative approach involves constructing a fusion protein that corresponds to a specific region of the target protein, such as the REJ domain, as a means to investigate the functions of polycystin-1. Moreover, a variety of systems for expressing fusion proteins can be employed, whether using prokaryotic or eukaryotic cells (Hayat et al., 2018). One particularly convenient and versatile method for producing, purifying, and identifying fusion proteins in *E.coli* involves the utilization of the His gene fusion system (Pimienta et al., 2019; Minkneret al., 2020).

Recognizing the need for a rapid and well-defined process that can yield abundant quantities of recombinant proteins, we have devised an efficient protocol. In response to this necessity, we integrated our gene cloning into the pET-21-MPB vector. Our objective in doing so was to optimize the expression of the REJ gene and enhance the overall yield. The prospect of enhancing the production of recombinant human proteins in eukaryotic hosts, given their inherent complexity and continuous expression, is indeed an attractive notion.

Recombinant soluble protein production is a challenge in biotechnology and the method that can aid in increasing protein solubility is using tag fusions in N/C-terminal like maltose binding protein (MBP). Tag fusions are protein segments that are produced along with the target protein and their expression enhances protein solubility, yield, expression, and efficacy (Sun et al., 2013).

Based on the aforementioned, this study is designed to demonstrate, the prokaryotic expression system of two strains of *E. coli* (Mach-1 and BL21). Also, to enhance protein solubility by MBP partner. The fusion protein was employed to investigate the interaction of PC-1 with ECM proteins, which is a crucial step in understanding the protein function and identification of relevant biological pathways.

2. Materials and methods

2.1. Materials

The pET21a(+), and pET21a(+)-MBP plasmids were purchased from Addgene (USA). Restriction enzymes XhOI, BamHI, HindIII and T4 DNA ligase were obtained from New England Biolabs (USA). Escherichia coli (E. coli) Mach-1 and BL21-DE3 cells for protein induction were kindly provided by Dr. Ahmed Atef at King Abdulaziz University, Faculty of Science, Biology Department.

2.2. Methods

2.2.1. Cloning of His-REJ into pET-21a (+)-maltose binding protein expression vector

Using the sequence retrieved from GenBank under accession number AAC37576.1, we synthesized residues spanning genes 2151 to 2451 through the phosphoramidite synthetic method. In this study, DNA synthesis was employed instead of the conventional amplification approach, as the REJ gene proved challenging to amplify using standard PCR techniques. Consequently, the REJ gene sequence was designed and synthesized by Synbio (USA), followed by cloning into a pUC57-Amp cloning vector. Notably, BamHI and XhoI restriction sites were introduced at the 5' and 3' ends of the sequences, respectively, to facilitate subsequent cloning steps. Detailed information regarding the cloning, amplification, and sub-cloning of the REJ gene into the PET-21a (+) expression vector can be found in our prior publication (Sonbol& AlRashidi, (2022).

The pET-21(+) expression vectors were utilized to induce the REJ fusion protein as His tagged soluble protein with maltose binding protein tag. pUC 57-REJ was used as a template DNA. Primers were designed to generate a 300 bp by using the NCBI primer designing tool. All oligonucleotides used were synthesized by macrogen (Korea). Forward and reverse primers were: 5´AATTAAGCTTTCCGGGTGCGAACATCTGCCT-3´, (T7 terminator) 5´-GCTAGTTATTGCTCAGCGG-3´respectivly. The Forward primer containing HindIII restrictions site was shown in bold underline.

2.2.2. PCR amplification of the pET-21a(+)-REJ gene fragments

The PCR reaction was performed in a MultiGene thermal cycler (Labent, USA). The amplification conditions were as follows: 4 min and 10 sec at 94 °C, followed by 32 cycles of

94 °C for 30 sec, 48 °C for 30 sec, 72 °C for 1 min, and a final extension at 72 °C for 10 minutes. The PCR reaction contained the forward and reverse primer, (2 µl), pUC 57-REJ plasmid (1 µl), GoTaq master mix (25 µl) and deionized water (20 µl). The PCR product was analyzed by running the samples on a standard agarose gel which was performed at room temperature using a 0.5% (w/v). The PCR product was purified by a QIAquick PCR Purification kit according to the manufacturer's instructions.

2.2.3. Restriction of the pET-21a(+)-MBP and PCR product

Double digestion reactions were performed for both the PCR Product and pET-21a(+)-MBP expression vector. pET-21a (+)-MBP and PCR product were cut with restriction enzymes HindIII and XhoI (New England Biolabs, USA). The products were then analyzed by agarose gel electrophoresis.

The digested REJ gene fragment was ligated to the previously digested expression vector pET-21a(+)-MBP by T4 DNA ligase [89]. The setup of the ligation reaction with the ratio of 1 to 3 (insert to vector). The reaction included: digested vector (2µl), PCR product (5 µl), DNA ligase (1 µl), 10 x Rapid ligation buffer (2 µl), deionized water (10 µl).

2.2.4. Transformation of pET-21a(+)-MBP-REJ recombinant DNA into competent E. coli Mach -1

All steps in this protocol were carried out under sterilized conditions. Mach-1 competent *E. coli* was used for the transformation reaction. *E. coli* mixed with recombinant pET-21a(+)-MBP-REJ. Cells then were heat-shocked in a 42 °C water bath for 40- 45 sec and then immediately transferred to ice for 2 min. Then, the SOC medium was mixed with the competent cell and incubated at 37 °C. After shaking for 1 h at 225- 250 rpm, the transformed cells from each tube were spread on a pre-warmed LB-agar/ ampicillin and incubated overnight at 37 °C. The next day, the colonies on each plate were counted to determine the efficiency of the transformation. Growth of white *E. coli* colonies on LB-agar / ampicillin plates indicated that only cells transformed with a plasmid bearing the antibiotic-resistant gene were grown. The plate was then stored at 4 °C.

2.2.5. Screening for positive clones and confirmation using colony PCR

The colony PCR test was performed to select a positive colony that contains recombinant DNA. The colony PCR procedure was done by selecting white colonies on a culture plate and mixing each of them with a PCR mixture (10 µl). The PCR reaction mixture was subjected to the following cycling conditions: 4 min and 10 sec at 94 °C, followed by 32 cycles of 94 °C for 30 sec, 48 °C for 30 sec, 72 °C for 1 min, and a final extension at 72 °C for 10 minutes. The reaction contained: T7 terminator reverse primer, (GCTAGTTATTGCTCAGCGG) (1 µl), AATTAAGCTTTCCGGGTGCGAACATCTGCCT (1 µl), GoTaq master mix (5 µl), DNA template (bacterial colony) and deionized water (3 µl)

2.2.6. Extraction and purification of recombinant DNA

The recombinant pET21a(+)-MBP-REJ was purified using the plasmid miniprep kit (Zymo Research, USA, Cat. No. D4208T). The purification method was performed according to the manufacturer's protocol.

Verification of the pET-21a(+)-MBP-REJ recombinant was made by the transformation into BL21-DE3 competent cells, PCR reaction and colony PCR. After plasmid mini-preparation, verification of the pE21-MBP-REJ plasmids was performed by PCR using a T7 terminator primer. The reaction constituents were as follows: AATTAAGCTTTCCGGGTGCGAACATCTGCCT (2 µl), T7 terminator reverse primer (GCTAGTTATTGCTCAGCGG) (2 µl), pET-21a(+)-MBP-REJ (1 µl), GoTaq master mix (25 µl) and Deionized water (20 µl). The PCR products were separated by electrophoresis on a 0.5% agarose gel.

The purified recombinant pET21a(+)-MBP-REJ DNA was sequenced at King Fahad Research Laboratory, Jeddah, KSA. The sequencing was performed using a 3500xL genetic analyzer (HITACHI, Japan). The sequence of pET-21a (+)-MBP-REJ was analyzed using the FinchTV software (V1.4.0). Then, it was compared with a theoretical REJ sequence via the European Bioinformatics Institute (EMBL) database.

The correct pET-21a(+)-MBP-REJ plasmids were extracted from Mach-1 strain using a miniprep kit (Zymo Research, USA, Cat. No. D4208T), then transformed into BL21-DE3 competent cells to be used in protein expression. After colony PCR one positive colony was picked and cultured in LB media (5 ml) supplemented by ampicillin (100 µg/ml) for 16-18 h in an orbital shaker (Witeg Wisd Shaking Incubator WIS-20, Germany) (220 rpm) at 37 ˚C).

2.2.7. REJ Fusion protein expression

The positive single colony was picked to inoculate the LB medium (5 ml) containing ampicillin (100 µg/ml). The tubes were then incubated overnight in an orbital shaker at a speed of 220 rpm at 37 °C. Then 2 ml from the cultured medium was used to inoculate TB medium (250 ml) (100 µg/ml ampicillin) in a sterile conical flask. Flasks were incubated overnight in an orbital shaker at a speed of 220 rpm at 37 °C. High-density cell growth in TB media is followed by spontaneous induction of protein expression. After 24 h the cells were then harvested by centrifugation at 6000 rpm using a refrigerated centrifuge (PrO-research by Scientific Ltd, UK) for 10 min at 4 °C. Then the supernatants were decanted, and the pellets were stored at –70°C. The pellet was rinsed once with native lysis buffer (50 mM NaH2PO4, 300 mM NaCl, and 10 mM imidazole), supplemented with 1mg/ml lysozyme. The mixture was incubated at 4 ºC on a shaker for 1 h. Then 20 % triton 100, and tween 20 were added to the resuspended pellets and sonicated using MSE Sanyo Soni-prep 150 sonicator (Sanyo, Japan) for 30 × 10 s cycles at 200– 300 W. Lysate was

centrifuged for 10 min at 6000 rpm at 4 °C. The supernatant was collected. The pellet was solubilized using denaturing lysis buffer (100 mM NaH2PO4, 10 mM Tris-HCl, and 6 M urea). The supernatant contains REJ-His fusion protein stored at -70 °C until needed. Samples from the supernatant (native), pellets (denature), and non-induced were analyzed using the reduced 15% SDS-PAGE.

2.2.8. Purification of the His-REJ fusion protein under native conditions using PureCube His affinity agarose

The His-REJ fusion protein was purified using immobilized metal affinity chromatography (IMAC) resins (PureCube Ni-NTA Agarose, Cube Biotech, Germany, Cat. No.600364-3026-027). The resin was resuspended, and 1 ml of 50% suspension was transferred to a centrifuge tube and then settled. Lysis buffer (NaH2PO 50 mM, NaCl 300 mM, Imidazole 10 mM) (2.5 ml) was added, and the slurry was gently resuspended. The cleared lysate (10 ml) was incubated with the equilibrated Ni-NTA agarose resin at 4 °C for 5 h on an end-over-end shaker. The binding suspension was moved to a gravity flow column with a capped bottom. The tube was rinsed with lysis buffer to remove adhered resin, and the flow-through was collected. The column was washed three times with 5 ml of native wash buffer (NaH2PO 50 mM, NaCl 300 mM, Imidazole 20 mM)). His-tagged protein was eluted in 0.5 ml of native elution buffer (NaH2PO 50 mM, NaCl 300 mM, Imidazole 50 mM), with each elute collected in separate tubes. Protein concentration in each fraction was assessed with 15% reduced SDS-PAGE. Purified protein was stored at -70°C.

Post-washing, elution (dissociation from the ligand and protein recovery) of the target protein from the ligand is achieved by altering the buffer conditions to disrupt the binding interactions between the target and the ligand. In this study, we identified the optimal buffer condition at pH 8. We achieved this by incubating REJ overnight with the support matrix containing the affinity ligand, facilitating the binding of REJ protein from the sample to the immobilized ligand. This ensured that all other molecules washed through the column. All five elution fractions were collected and contained 500 mM Imidazole.

3. Results

3.1 Cloning of His-REJ into pET-21a(+)- maltose binding protein expression vector

The sections of the PKD-1 gene responsible for the REJ domain's encoding were inserted into the pET-21a(+)-MBP expression vector. The detection of an approximately 300 bp band on a 0.5% agarose gel serves as an indicator of the presence of the REJ gene (Figure 1A). Using pUC75-REJ as the template, we employed reverse and forward primers to generate a 300 bp DNA fragment, which represents a portion of the coding region within the REJ gene.

The PCR product then becomes the target gene to be ligated into the pET-21a(+)-MBP expression vector after the PCR product is purified.

The pET-21a(+)-MBP vector underwent double digestion using the XhoI and HindIII restriction endonucleases, and the outcomes of this digestion process are depicted in Figure 1(B). The successful digestion of the pET-21a(+)-MBP vector is evident in Figure 1(B), lane 2. The linearized vector, as shown in the Figure, aligns with the size specified by the supplier (6600 bp), as indicated in lane 1. The pET-21a(+)-MBP expression vector has been specifically designed for the efficient and high-throughput purification of recombinant proteins on a large scale.

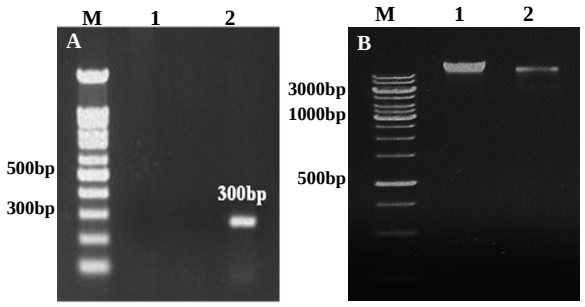

Figure (1): (A) 0.5% (w/v) agarose gel electrophoresis. Lane 1 represents a negative control; lane 2 is the REJ PCR product which has a 300 bp band. Lane M is the DNA ladder (100 bp). **(B)** The pET-21a(+)-MBP vector reveals the results of the XhoI and HindIII double digestion. In lane 1, the undigested vector, which appears at 6600 bp, is displayed alongside a 1kb ladder (M). In lane 2, the double digestion of the pET-21a(+)-MBP vector.

3.2. Transformation of recombinant DNA into competent E. coli Mach 1

When the fragment corresponding to REJ was ligated into pET-21a(+)-MBP expression vector and transformed into *E.coli* the recombinants containing the desired construct termed pET-21a(+)-MBP-REJ were identified following extraction and analysis of the plasmid DNA. The transformation was carried out by using the competent cell *E.coli* Mach-1 multiplied by the recombinant plasmid pET-21a(+)-MBP-REJ Figure 2.

Figure (2) displays the outcome of a successful positive colony PCR. Lanes 1 to 20 exhibit the expected bands, approximately 300 bp in size. For subsequent analysis, we chose lane number 16.

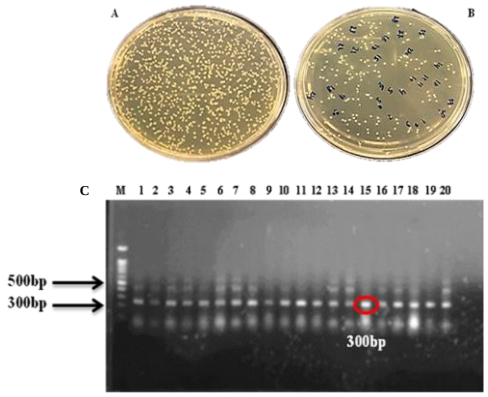

Figure (2): The image depicts two categories of competent cells and A 0.5% (w/v) agarose gel electrophoresis of the colony PCR. (A) represents Mach-1 cells, while (B) represents BL21-DE3 cells utilized to improve the successful expression of His-REJ. (C) The desired bands with a 300 bp can be seen in lanes 1- 20. The selected positive colony was number 16. M is the 100 bp DNA ladder.

The plasmid DNA extraction and purification from Mach-1 cells via miniprep resulted in the acquisition of pET-21a(+)-MBP-REJ recombinant DNA. Figure 3 (A) verified the correct size of the pET-21a(+)-MBP-REJ recombinant plasmid, which was 6900 bp. This result aligns with the data generated by the SnapGene software.

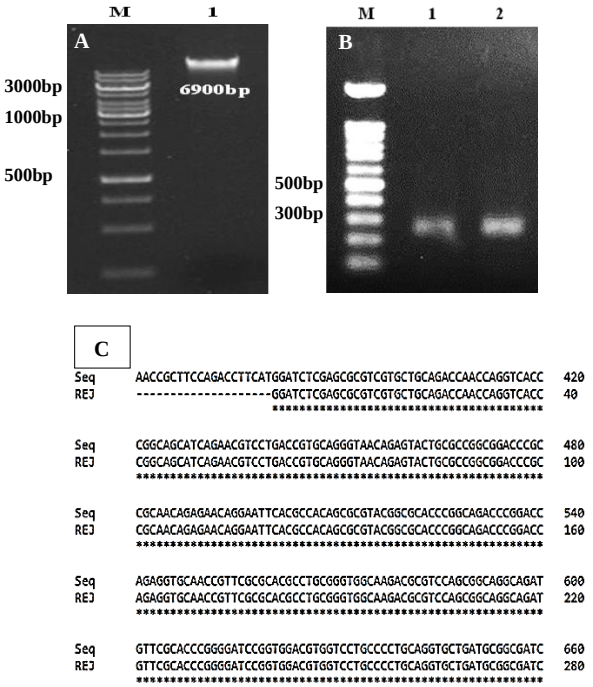

Figure (3): (A). 0.5% (w/v) agarose gel electrophoresis displaying the recombinant pET-21a(+)-MBP-REJ is depicted in lane 1, showcasing a band at 6900 bp. M is the 1 kb DNA ladder. **(B).** The desired REJ gene inserted into the pET-21a(+)-MBP vector is shown in lines 1 and 2. M is a 100 bp DNA ladder. **(C)** The alignment between the pET-21a(+)-MBP -REJ obtained from sequencing and the theoretic (GenBank: AAC37576.1) REJ gene sequence. Differences are marked by a dot (.) and matches are marked with a star (*).

3.3. Verification of the pET-21a(+)-MBP-REJ recombinant DNA by transformation into BL21-DE3 competent cells, PCR and sequencing

Amplification of the pET-21a(+)-MBP-REJ recombinant DNA produced a ~ 300 bp PCR product which is shown in Figure 3 (B).

We performed a side-by-side analysis, as depicted in Figure 3 (C), where we compared the theoretical sequence of the REJ gene obtained through NCBI-BLAST with the sequencing outcomes derived from our research. In our laboratory's gel electrophoresis findings, we noted exact correspondences between the theoretical information and the sequencing data. In both cases, there was clear evidence of a ~300 bp segment within the REJ gene sequence. To validate this alignment further, we executed a sequence alignment between the theoretical REJ gene sequence and the PCR product, as shown in Figure 3.

3.4. Verification of the correct pET-21a(+)-MBP-REJ recombinant DNA by transformation of the pET-21a(+)-MBP-REJ plasmids into BL21-DE3 competent cells

The colony PCR results in Figure 4 (A) indicated the presence of a 300 bp fragment. This indicates that cloning of the fragment encoding PKD-1 REJ into pET-21a(+)-MBP expression vector was successful. The result of extraction and purification of the positive colonies containing recombinant REJ is presented in Figure 4 (B). The pET-21a(+)-MBP-REJ recombinant DNA have the same length (6900 pb) as pET-21a(+)-MBP-REJ (extracted from Mach-1 strain).

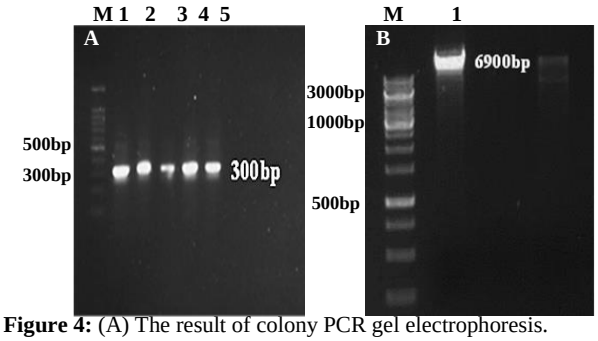

Figure 4: (A) The result of colony PCR gel electrophoresis. Lanes 1-5 represent the 300 bp REJ gene bands. M is the 100bp DNA ladder. (B) 0.5% (w/v) agarose gel electrophoresis. Lane 1 is the recombinant DNA pET-21a(+)-MBP-REJ extracted from BL21-DE3 competent cells. M is the 1 kb DNA ladder.

The result of PCR amplification of the correct pET-21a(+)-MBP-REJ plasmid exhibited a ~ 300 bp segment of the REJ

gene. This also indicated the successful cloning of the REJ gene in the expression vector Figure 5.

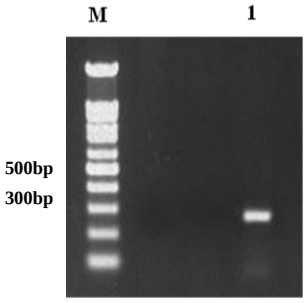

Figure 5: A 0.5% (w/v) agarose gel electrophoresis. Lane 1 desired the REJ gene. M is the 100 bp DNA ladder.

3.5. Fusion protein expression

Figure 6 (A) displays the presence of the soluble His-REJ fusion protein. The appearance of a 53 kDa band in lane 3 (comprising 13 kDa of His-REJ and a 40 kDa MBP tag) corresponds to the His-REJ fusion protein. The optimization process involved an overnight induction time at a temperature of 37ºC. Figure 6 (B) shows the SDS-PAGE analysis performed on the His-REJ fusion protein under reducing conditions, validating the successful expression of the fusion protein. In Lane 3, the soluble His-REJ protein within the native fraction is observed. Furthermore, the His-REJ fusion protein is also visible in the insoluble fraction (Lane 2 and Lane 1) depicted in the non-induced fraction.

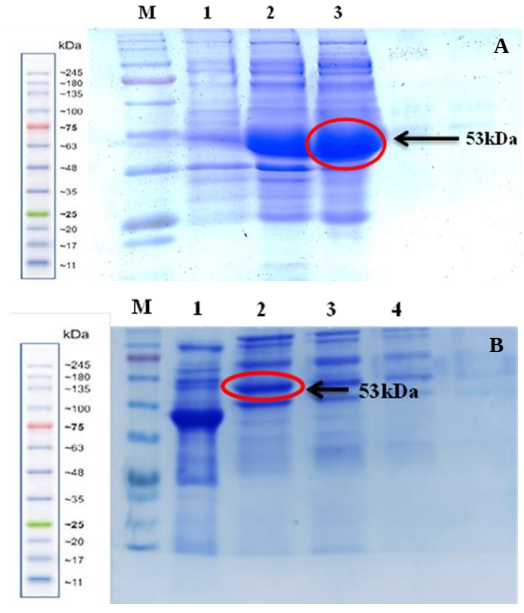

Figure 6: A 15% (w/v) reduced SDS-PAGE of the expressed His-REJ proteins. Lane 1 shows the non-induced fraction, and Lane 2 represents the pellet fraction of His REJ. Lane 3 presents the soluble His-REJ protein in the native fraction. Lane M presents the molecular weight protein marker. **(B)** The purified His-REJ by Ni-NTA affinity chromatography first round. Lane 1 is the His-REJ supernatant. Lanes 2, 3 and 4 are successive elutions showing the affinity purified His-REJ at 53 kDa. Lane M presents the molecular weight protein marker.

Figure 7 shows the outcome of purifying the His-REJ fusion protein by IMAC chromatography second round, which was validated by observing a band in lane 1 with a molecular weight of approximately 53 kDa. We found an optimum buffer condition at pH 8 and incubated REJ overnight with the support matrix containing Imidazole 500 mM for elution.

The soluble fraction containing the MBP-REJ fusion protein was subjected to purification through affinity chromatography. The outcomes of this purification process are depicted in Figure 7, where the band at 53 kDa corresponds to the MBP-REJ fusion protein. Subsequently, various concentrations of the MBP-REJ fusion protein (0.5, 1.5, 2.5, 3, and 3.7 mg/ml) were quantified using both the BCA kit and Nanodrop.

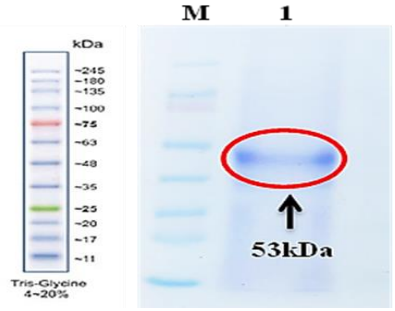

Figure 7: A 15% (w/v) SDS-PAGE of the purified MBP-REJ fusion proteins in Lane 1 that present in the soluble fraction corresponding to 53 kDa, Lane M is the molecular weight protein marker.

4. Discussion

The expression of full-length recombinant PC-1 has confirmed that the protein has a predicted mass of approximately 460 kDa and a glycosylated mass of 520 kDa (Courtney et al., 2021). The REJ domain which is a part of the extracellular domain has a vital role in cell recognition and cell-cell and cell-matrix adhesion (Streets et al., 2009; Qian et al., 2005). Inspecting different systems to assess the expression of PC-1 as recombinant proteins is crucial due to the challenges associated with its purification and the low yield (Weston, et al., 2003). Alternatively, the construction of a fusion protein that corresponds to a specific region of the target protein, such as the REJ domain, can provide a way to study PC-1 functions.

Natural sources have limitations in providing sufficient quantities of proteins, posing challenges in extraction and cost-effectiveness. In contrast, genetic engineering methods enable cells to produce ample amounts of proteins, including foreign ones, which can be purified for various applications in research, industry, and therapeutics (Geisse & Fux, 2009; Ki & Pack, 2020). *E. coli* is a preferred choice for heterologous protein expression due to its ease of use, rapid growth, high-density cultivation capability, and efficient production of

recombinant proteins using established genetic and molecular methods (Ki & Pack, 2020). The *E.coli* expression system has drawbacks in biopharmaceutical applications, such as lacking eukaryotic post-translational modifications, low solubility, and improper folding. Nonetheless, biotechnological advancements are making *E. coli* a viable choice for protein expression (Ki & Pack, 2020). Affinity tags aid in target protein purification, while enhancing protein solubility with fusion tags is a research focus, though effects can vary (Köppl, et al., 2022).

A recent study successfully optimized the expression conditions by inducing REJ-His fusion protein expression at 25℃ for 6 hs with 1 mM IPTG. Unfortunately, their protein was observed in insoluble fractions (Sonbol & AlRashidi, 2022). Previously, it has been noted that when the molecular weight of proteins is less than 30 kDa, they frequently need to be combined with substances that enhance their solubility to be successfully produced in a soluble state (Hasegawa et al., 2002).

Furthermore, this study represents a pioneering effort in exploring the functions and mechanisms of previously unknown REJ proteins. To understand the structure and function of REJ, we utilized synthetic nucleotides for the production of the REJ-His fusion protein (Sonbol & AlRashidi, 2022). Since many functions of REJ proteins remain unidentified, obtaining sufficient quantities of pure protein is crucial for detailed biophysical and biochemical studies (Rosano et al., 2019). To achieve the desired product and ensure alignment with the theoretically proposed protein sequence, we designed specific REJ nucleotide sequences. These sequences were sent to Synbio in the United States, where a partial-length gene (300 bp) was synthesized, incorporating the essential exon regions while maintaining sequence integrity, following a similar approach by Tan et al. (20212).

This strategy allowed us to create a synthetic gene that closely resembled the proposed product, overcoming the challenges associated with the complex exon-intron organization observed in the REJ protein across different exons. We successfully cloned and expressed the REJ protein using the DNA synthesis method instead of the conventional methodology (Sonbol & AlRashidi, 2022). To obtain the theoretical sequence of the REJ gene, a comparison was performed between the REJ domain of PC-1 in the NCBI-BLAST database (GenBank: AAC37576.1) and the sequencing result obtained in our laboratory. The alignment of the theoretical REJ gene sequence with the REJ sequence derived from the laboratory`s sequencing reaction revealed a significant similarity of 98% between the recombinant REJ protein and the obtained sequencing result. These results confirm the accurate reproduction of the REJ gene sequence through the DNA synthesis method and validate the successful expression of the REJ protein in our study.

According to Gholami et al. (2018), the expression of the recombinant forms might be enhanced upon the use of eukaryotic hosts because the structure of many human proteins is comparatively complicated. While, Schröder et al. (2011), found that *E. coli* could express a small segment of molecular weight of an expressed REJ protein that contains a considerable number of β-sheets and is possibly similar to fibronectin type III. Therefore, their study suggested that the REJ is not a single domain ~~[10]~~. Moreover, using BL21-DE3 competent cells to transform the recombinant REJ, guarantees the efficiency of more than 60% of the other competent cells (Xing et al., 2021; Lozano Terol et al., (2021).

It is with great satisfaction that the successful cloning and expression of the synthesized human REJ gene using a straightforward implementation procedure is reported. This procedure has allowed for the generation of substantial quantities of recombinant protein. The main objective of this study was to produce a soluble form of the protein, as protein solubility plays a vital role in its expression, function, and proper folding. Soluble fusion proteins hold significant potential for therapeutic applications due to their improved solubility, which facilitates easier handling, purification, and formulation. As a result, they represent promising candidates for further development as therapeutic agents (Chen, et al., 2023; Hasan et al., 2022; Grund et al., 2021).

Membrane proteins are proteins that pose challenges in expression but have emerged as highly valuable targets due to their therapeutic potential. These proteins, which are difficult to produce, have become a primary focus in the field of therapeutics. Membrane proteins are integral to cell membranes and are involved in important functions such as signalling and transport. Despite the challenges associated with expressing and studying these proteins, their significance in therapeutic contexts has driven extensive research efforts to overcome these obstacles and fully utilize their potential benefits (Bian et al., 2022; Lloyd et al., 2021; Xu et al., 2013).

In the realm of protein biotechnology, it is a common practice to utilize large soluble fusion partners to improve the yield and solubility of recombinant proteins (Ren et al. (2022). In our study, we employed the MBP tag as an example of a fusion partner. Interestingly, the use of the MBP tag has been found to significantly enhance the overall production of the fused proteins (Ren et al. (2022). Moreover, Reuten et al. (2016) emphasized that the fusion of MBP with proteins consistently resulted in the most significant increase in protein production when compared to commonly used tags such as Glutathione S-transferase (GST) (Reuten et al., 2016). In addition, Nemergut, et al. (2021), utilized the MBP tag not only to improve the purification of the fusion protein via affinity chromatography but also to detect the target protein. In our study, we employed MBP as a chaperone by fusing it with the recombinant REJ protein. This fusion approach was utilized to eliminate the requirement for additional steps such as solubilization and refolding during the protein purification process. Our results demonstrated a powerful experimental approach to further study of the solubility, function and REJ- ECM interactions of proteins, also it should pave the way to systematically characterize the effects of disease-causing mutations in the REJ module of human PC-1.

We reported a successful expression of REJ-His fusion protein as a soluble protein using the MBP tag because of the following reasons: Firstly, the MBP protein's primary structure indicated that it represents an ideal partner for fusion protein expression in mammalian cells, especially for secreted targets. Despite having a substantial molecular weight of around 40 kDa, MBP exhibits the ability to enhance the solubility of fusion proteins. Furthermore, MBP is characterized by its globular structure and abundance of α-helices, making it an excellent candidate for molecular replacement (Zhang et al., 2021; Greenfield et al., 2020).

These findings were consistent with our observations, of successful fusion of MBP with the REJ protein, resulting in the formation of a 53 kDa MBP-REJ fusion protein. Secondly, it is worth noting that the expression level is significantly influenced by temperature. Several studies have demonstrated that reducing the induction temperature to 25°C and 18°C can substantially increase the expression levels by 80% and 60%, respectively (Kaur et al., 2018). Conversely, other research has emphasized the use of an induction temperature of 37°C, where the incorporation of MBP tags has shown solubility enhancement of over 60% and prevention of inclusion body formation (Nguyen, et al., 2019). Notably, our findings align with the latter approach, supporting the efficacy of using 37°C as the induction temperature for optimal protein expression while utilizing MBP tags to improve solubility. Thirdly, in contrast, Fathi-Roudsari et al. (2018) found that *E.coli* strains Rosetta- gami (DE3) (a derivative of BL21 (DE3) had the highest level of soluble reteplase production in comparison to *E.coli* strains BL21 (DE3) and Shuffle T7.

In this study, we employed two different *E. coli* strains, namely BL21 (DE3) and Mach-1, to investigate the production of MBP-REJ fusion protein. Through our experimentation, we determined that the optimal conditions for achieving high protein yield involved auto-inducing the culture followed by incubation at 37 °C overnight. Our results indicated that BL21 (DE3) strain exhibited superior performance in terms of protein production, corroborating the results reported in a comparable investigation (Quartinello, et al., 2023; Shafique, et al., 2022).

Indeed, the using of auto-induction represented a method for the high-yield growth of bacterial cells and higher expression of recombinant proteins, which was created by Studier. This technique provides a high expression level and offers some advantages over IPTG induction, including the ability to mitigate issues such as protein misfolding, costs have been lowered significantly, and insolubility protein production (Fathi-Roudsari et al., 2018; Gaglione, et al., 2019; Blommel et al., 2007). This was similar to our study.

Based on Cheung et al. (2012) reported that the pET-21a(+)-MBP expression vector contains a fusion tag that consists of a tandem repeat of polyhistidine (His6-tag), and can bind to an affinity matrix comprising metal ions for protein purification (Babbal et al., 2022; Srivastava et al., 2019). Our MBP-REJ fusion protein purification was successfully achieved by subjecting the supernatant containing the expressed MBP-tagged REJ in *E.coli* to immobilized metal affinity chromatography (IMAC) using a nickel-charged column to capture the His6 tag at the N-terminus of each hybrid construct. Where the proteins bound to the column were then eluted with imidazole to release the His6-tagged fusion protein. Our first round of IMAC was intended to separate the MBP-REJ from a mixture, while the second round was to capture the MBP-REJ fusion protein tagged His (Andreeva et al., 2021; Riguero et al., 2020).

The functionality of the protein is not compromised by the presence of the MBP tags. This was demonstrated in a study by Guo et al. (2018) in which they successfully produced bioactive leukaemia inhibitory factor (LIF) by fusing it with MBP, resulting in the formation of the MBP-LIF fusion protein. They utilized the *E.coli* system for protein expression and employed a one-step purification method using gravitational affinity chromatography. The purified MBP-LIF products demonstrated the ability to selectively inhibit the growth of M1 cells, and this inhibition showed a dose-dependent pattern. Importantly, their findings indicated that the presence of the MBP tag in the MBP-LIF fusion protein did not hinder the bioactivity of mLIF ~~[57]~~.

5. Conclusions

In conclusion, we successfully cloned and expressed the human REJ synthesized gene. The production of soluble protein allowed us to produce large quantities of recombinant protein. Protein solubility was necessary for protein expression, function, and folding; therefore, we used the MBP tag to produce REJ protein in the soluble form. Our results demonstrated a powerful experimental approach to further studying the function of REJ proteins and should pave the way to systematically characterizing the effects of disease-causing mutations in the REJ module of human PC-1.

Author Contributions:

Conceptualizing the study, formulating the research question, designing the experimental framework, developing the hypothesis, outlining the methodology and supervising the laboratory work, writing the paper, H.S.S. Execution of laboratory experiments, data collection, data analysis, diligently following the experimental protocols and ensuring the accurate and reliable collection of data, statistical analysis, and the interpretation of results, writing the paper, A. A. A.

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