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Research article

Optimization of expression and purification of recombinant Apollo *(SNM1B)* protein using affinity chromatography

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Abstract. Apollo, also referred to as SNM1B, is a nuclease involved in telomere stability and DNA repair, and its dysfunction is associated with the development of a number of pathologies, including hereditary kidney cancer. The growing interest to this protein is due to its possible use as a target for antitumor therapy. The present study focuses on the optimization of the expression and isolation of the homogenous recombinant Apollo (SNM1B) WT protein by means of affinity chromatography purification. Expression vector constructs pET28c-hSNM1BcoCut and pETHSUL-hSNM1BcoCut were used to produce the protein after their transformation into *Escherichia coli (E. coli)* Rosetta2 (DE3) and NovaXG strains. Variation in the induction parameters such as concentration of IPTG, incubation temperature, presence of detergent NP-40, glycerol, urea and duration of the post-induction incubations all were subjected to rigorous testing, in order to ascertain the optimal conditions for the obtaining soluble recombinant proteins. The purification process was conducted through the utilization of Ni-NTA affinity chromatography. The purity and solubility of the protein were evaluated by SDS-PAGE. The obtained data allowed to determine effective conditions for producing the soluble and highly homogenous Apollo protein suitable for further studies, such as protein-protein interaction, DNA binding, immunization to obtain specific antibodies, and utilization for screening of inhibitors for cancer therapy.
Keywords: DNA repair, Apollo, SNM1B, renal cell carcinoma, DNA damage, TRF2 protein

Introduction

The stability of the human genome is constantly challenged by damage to cellular DNA by various endogenous and exogenous sources. These include normal by-products of oxidative

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metabolism, UV and ionizing radiation, chemical mutagens, and viral infectious agents. DNA damage such as double-strand breaks, interstrand DNA cross-links, depurination, and deamination can cause genetic mutations and ultimately compromise genome integrity and cellular homeostasis [1-3]. To preserve genetic stability and ensure the accurate transfer of hereditary information, cells employ multiple DNA repair pathways to remove lesions from cellular genome. These encompass excision repair, homologous recombination, non-homologous end joining (NHEJ), and specialized mechanisms responsible for the removal of interstrand DNA cross-links (ICL). Central to these processes are numerous enzymes exhibiting nuclease, helicase, and ligase activities, which cooperate to restore DNA structure and function [4-8].

Among them, Apollo, encoded by the *SNM1B* gene, belongs to the SNM1 family of DNA nucleases and performs a 5'→3' exonuclease function that is essential for proper DNA end resection during replication, telomere protection, double-strand break (DSB) repair, and ICL unhooking [9-12]. Similar to other SNM1 family, Apollo contains a β-CASP domain that forms the enzyme's catalytic center. This structural domain binds divalent metal ions, most commonly Zn^{2+}, which are required for phosphodiester bond cleavage. The β-CASP domain is therefore critical for Apollo's nuclease function, ensuring accurate substrate interaction and efficient catalysis [13-15].

Apollo has an essential role in the protection of telomeric DNA from its misrecognition as a DSB site by the repair system. This is achieved by Apollo interacting with components of the "shelterin" telomere complex, in particular the TRF2 protein. Apollo is involved in the replication of the telomere leader chain. It promotes the formation of the protective telomere structure, the T-loop. Loss of SNM1B function or disruption of its expression can lead to telomere shortening, chromosomal instability and chromosome fragmentation, which may be associated with proliferative and oncological diseases [16-18].

The data collected to date suggest that Apollo *(SNM1B)* may be involved in the pathogenesis of a number of different types of cancer, including kidney cancer, particularly the renal cell carcinoma (RCC). RCC is characterized by high genetic heterogeneity, alterations in cell cycle regulation, apoptosis and DNA damage response and is the most common form of kidney cancer [19]. Although mutations in *SNM1B* are relatively rare in the tumor genome, transcriptomic studies suggest that the expression of *SNM1B* is suppressed or abnormally regulated in certain subtypes of RCC, in particular in tumors with marked telomere dysfunction and high levels of chromosomal instability. These data suggest that functional *SNM1B* deficiency may be involved in tumor progression through accumulation of mutations and genomic rearrangements [20,21].

The additional importance of Apollo (*SNM1B*) in carcinogenesis is underlined by its role in the elimination of ICLs, one of the most cytotoxic types of DNA damage, which occurs particularly under the action of chemotherapeutic agents such as cisplatin and mitomycin C. This function makes Apollo a potential target for anticancer therapy: reducing *SNM1B* activity may increase the sensitivity of tumor cells to drugs, whereas in healthy tissues its function is critical to protect normal cells against genotoxic damage [22-25].

Although the Apollo protein is of great scientific interest, it is difficult to characterize its repair activities *in vitro.* This is due to the problems associated with obtaining the soluble protein in recombinant form. The Apollo protein, like many other eukaryotic enzymes, has a low solubility, a tendency to aggregate, the formation of inclusion bodies in the prokaryotic expression system of *Escherichia coli,* and a susceptibility to proteolysis and denaturation. Another obstacle is the toxicity of the *SNM1B* gene product to the host bacterial cell, since the recombinant protein can cleave bacterial DNA when overexpressed, especially in the absence of controlled regulation of expression and translation [26-30]. Therefore, optimization of the expression of *SNM1B* gene product in the *Escherichia coli* system becomes a high priority, including: selection among available host strains (e.g. BL21(DE3), Rosetta, C41); optimization of induction conditions

(temperature, time, IPTG concentration); construction of the expression vector taking into account rare codons and accessibility of the translation initiation site; addition of soluble fusion tags (MBP, GST, Trx, SUMO); modulation of expression using weak or inducible promoters (e.g. Lac, T7).

Materials and research methods

Molecular cloning, expression, and purification of recombinant proteins

In this study, two commercially available Escherichia coli strains were used for cloning and protein expression. For plasmid construction and propagation, the *E. coli* strain NovaXG purchased from Novagen (Merck Chimie SAS, Fontenay sous Bois, France), was used for plasmid construction because of its recombination-deficient and endonuclease-deficient background, which facilitates high-yield plasmid DNA preparation. The Rosetta 2 (DE3) strain purchased from Millipore was used for recombinant protein expression. This strain is engineered to enhance the expression of eukaryotic proteins by supplying tRNAs for rare codons. It also contains the DE3 lysogen for T7 RNA polymerase expression.

To express the codon-optimized *SNM1B* gene (Apollo) in a prokaryotic expression system, a pETHSUL/hSNM1B plasmid construct containing an *hSNM1B* fragment with an N-terminal His6 tag was used for subsequent protein purification by affinity chromatography. The construct was assembled using standard molecular cloning techniques and verified by DNA sequencing. The hSNM1B gene insertion started with an ATG codon followed by a site encoding a poly-histidine tag: ATGGGTCATCATCACCATCATCATCATCAC (corresponding to the His6 tag). The nucleotide sequence of the insert (from the expression site to the stop codon) was 1983 nucleotides and included a codon optimized version of *hSNM1B* designed with *E. coli* codon preferences and a terminator sequence (lower case). The construct (pET-type vectors) contained EcoRI and BamHI restriction sites flanking the hSNM1B gene. For mutagenesis and subcloning of *hSNM1B* coding DNA sequence (cDNA), several primer sets were designed and used: N246I, Y273H (a point mutation in which tyrosine at position 273 is replaced by histidine). These amino acid substitutions in the Apollo protein sequence will be important for studying the function of the protein and for creating mutant variants. To introduce these mutations, oligonucleotide primers are used Fw-SNM1B-N246I d(CATGCTGCGTTGG**AT**CCAGACCCACCCTACG) and Fw-SNM1B-Y273H d(CCACGTCATCCCT**C**ACTCTGACCATTCCTC), which ensure the introduction of specific changes in the coding sequence of the *SNM1B* gene.

The first step of site-directed mutagenesis is to perform PCR in the presence of the following reagents: 1 x PfuUltra Buffer, 0.25 μM of primers, 200 μM of each dNTP, 1-25 ng matrix DNA, 2.5 U of PfuUltra high-fidelity DNA polymerase, and nuclease-free water. The fully mixed reagents were transferred to a thermocycler. Thermocycling conditions for PCR: denaturation at 98°C - 5 min, 22 cycles of amplification at 98°C - 30 sec, 59°C (depending on primers used) – 10-30 sec, 72°C - 30 sec per kb (depending on the length of cDNA) and final elongation at 72°C for 10 min, followed by a pause at 4°C.

The second step involves DpnI (KLD) treatment. 10μl of PCR product is incubated in a tube containing 1 x reaction buffer and 1U of DpnI. Cleavage of the PCR product by DpnI is an important part of the work. DpnI cuts only a methylated site, cleaving the template plasmid but not the newly synthesized PCR-generated product, which allows selection of transformed cells with a plasmid that has undergone site-directed mutagenesis.

Preparation of competent cells

To prepare competent cells, single colony from fresh plate was inoculated in 30 ml of S.O.C. (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM $MgSO_4$ and 20 mM glucose) and grown overnight at 37°C 200 rpm until OD_{600} ~ 0.4. The following steps

were carried out at 4°C. Cell culture was centrifuged at 4000 rpm for 20 min at 4°C. Then the cell pellet was washed with 0.5 Vol of sterile 10% glycerol. After centrifugation at 4000 rpm, 20 min, 4°C, the cell pellet was washed with 0.5 Vol of sterile 10% glycerol. The next centrifugation step is followed by washing of the pellet with 0.1 Vol 10% glycerol. After the last centrifugation, 0.5-1.0 ml of sterile 10% glycerol was used to resuspend the cell pellet. Cell suspension was aliquoted by 100 µL in pre-cooled 1.5 ml Eppendorf tubes, which were frozen immediately in dry ice and stored at -80°C.

Transformation of E. coli cells

For protein expression, *E. coli* Rosetta 2 (DE3) chemically competent cells (Millipore, Cat. No. 71397-0.2ML) were used for heat shock transformation. Plasmid DNA (pET28c-hSNM1BcoCut, 84 ng/µl; pETHSUL-hSNM1BcoCut, 75 ng/µl) was added to 50 µl of thawed competent cells on ice and gently mixed. After incubation on ice for 30 minutes, cells were heat-shocked at 42°C for 45 seconds, then immediately placed back on ice for 2 minutes. 450 µl of SOC medium was added, and cells were incubated at 37°C with shaking at 200 rpm for 1 hour. Following recovery, 100-200 µl of cell suspension was plated on pre-warmed LB agar plates containing appropriate antibiotics and incubated overnight at 37°C.

Electroporation NovaXG was purchased from Novagen (Merck Chimie SAS, Fontenay sous Bois, France). For plasmid propagation, electroporation was performed using E. coli NovaXG electrocompetent cells. Plasmids (pET28c/hSNM1BcoCut and pETHSUL-hSNM1BcoCut) were added (50-100 ng) to 25 µl of competent cells on ice and transferred into a chilled 1 mm-gap electroporation cuvette. Electroporation was carried out using a Gene Pulser (Bio-Rad) under the following conditions: 1.8 kV, 200 Ω, 25 µF. Immediately after electroporation, 350 µl of SOC medium (37 °C) was added to the cuvette. The cells were gently mixed, transferred to a sterile tube, and incubated at 37 °C with shaking (200 rpm) for 1 hour before plating on LB agar containing antibiotics.

Expression and purification of the hSNM1BcoCut WT protein

E. coli Rosetta2 (DE3) cells (Millipore, 71397-0.2ML) transformed with the expression plasmids pET28c/hSNM1BcoCut WT and pETHSUL/hSNM1BcoCut WT were grown in 2L LB medium supplemented with 50 µg/ml kanamycin or ampicillin at 30°C with vigorous shaking until the optical density at 600 nm (OD_{600}) reached 0.6-0.8. The temperature was then reduced to 14°C, and protein expression was induced by the addition of 0.2 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). The 2L cultures were incubated for an additional 16 hours at 14°C. all subsequent purification steps were carried out at 4°C. Bacterial cells were harvested by centrifugation and lysed using a French press at 18 000 psi in 50 mL lysis buffer containing 20 mM HEPES-NaOH (pH=8.0), 500 mM NaCl, 10 mM Urea, and 0.025% Nonidet P-40, supplemented with a Complete™ protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). Cell lysates were clarified by centrifugation at 30 000 × g for 1 hour at 4°C. The 40 mL supernatant was adjusted to 500 mM NaCl and 30 mM imidazole, then loaded onto a HiTrap Chelating HP column (GE Healthcare, Chicago, IL) at a flow rate of 0.5 mL/min. Bound proteins were eluted with a 15 mL 50-800 mM NaCl gradient in Buffer B2 (20 mM HEPES-NaOH, pH 8.0, 1000 mM NaCl, 10% Nonidet P-40) at a flow rate of 0.5 mL/min. The eluted fractions (0.5 mL) were analyzed by SDS-PAGE, and fractions containing pure *hSNM1BcoCut WT* protein were pooled and stored at -80°C in 50% glycerol.

Bradford Assay for Determining Protein Concentration

To quantify the protein concentration of Apollo fractions using bovine serum albumin (BSA) as a standard. BSA stock solution (1 mg/ml) was prepared by diluting 2 µl of 50 mg/ml BSA in 98 µl PBS. This standard was used to prepare a calibration curve by mixing 1-5 µl of 1 µg/µl BSA with 900 µl of PBS and 100 µl of 10x Bradford reagent (Bio-Rad, US), resulting in final concentrations of 1-5 µl of BSA per sample. A blank control was prepared by mixing 900 µl of PBS with 100 µl of 10x Bradford reagent. For experimental samples, 2 or 4 µl of protein was added to 900 µl of PBS, followed by 100 µl of 10x Bradford reagent. All tubes were inverted 10 times to ensure proper mixing and incubated at room temperature for 5 minutes. The solutions were then transferred into 1 ml plastic cuvettes.

The spectrophotometer (Eppendorf BioPhotometer, Germany) was calibrated using the blank control. Then, the absorbance of standard and sample solutions was measured at 595 nm. Based on the absorbance of standard values of BSA standards, an average calibration factor of 0.115 A_{595}/µg was calculated and used to determine protein concentrations in the experiment samples.

Protein analysis by SDS-PAGE

SDS-PAGE was performed using an XCell4 SureLock Midi system (Invitrogen, USA) with a 1 mm thickness on a 10% Bistris Midi Protein Gel (for pure protein fractions) or 4-12% Bistris Midi Protein Gel (for the fractions recovered during chromatography). NuPAGE MES SDS (Invitrogen, USA) was used to premix the running buffer. Four times sample buffer (Invitrogen, USA) and reducing agent, if necessary, were diluted to create the samples, which were then incubated for ten minutes at 75°C. Each well received the equivalent of 20-25 µg of protein for normalized uniform loading. Following incubation in 100 ml of Coomassie solution (0.1% Coomassie R-250 in 40% ethanol, 10% acetic acid), the electrophoresis gel was carefully heated in the oven and then gently shaken on an orbital shaker for 15 minutes at room temperature. The stain was then decanted, and deionized water was used to rinse the gel once. The gel was heated in a destaining solution (10% ethanol and 7.5% acetic acid) to produce the desired background.

Preparation of Cy5-labelled Oligonucleotides

To track enzymatic cleavage, oligonucleotides were tagged with the fluorescent dye Cy5 attached to their 3' ends. Following the labeling processes. The labelled oligonucleotides were purified on a Sephadex G-25 column equilibrated with water. Oligonucleotide duplexes were constructed by annealing the non-radioactively labelled strand in a solution containing 20 mM HEPES-KOH, pH 7.6, and 50mM KCl. The mixture was heated at 80°C for 5 minutes before gradually cooling to 4°C. Cy5-labelled oligonucleotide (10µM) cALi-1403-Cy5 and complementary oligonucleotide. Annealed duplex was kept in small aliquots at -20°C to avoid freeze-thaw cycles.

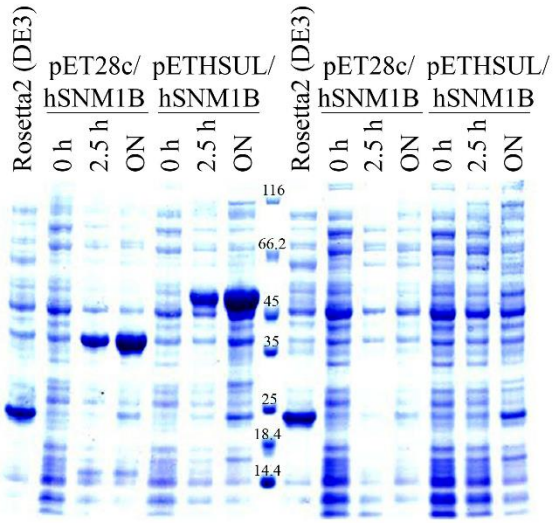
Results

The pET28c-hSNM1B and pETHSUL/hSNM1B expression vectors were transformed into *E. coli* NovaXG to purify recombinant plasmid DNAs. Plasmid DNA was isolated according to the GeneJET plasmid MidiPrep Kit (Thermo Scientific) protocol, and the transformants were verified for the presence of recombinant plasmids by restriction digestion and PCR analysis.

Subsequently, the plasmids were transformed into *E. coli* strain Rosetta2 (DE3), which carries a chromosomal copy of the gene encoding the T7 RNA polymerase under the control of

the lacUV5 promoter. Rosetta2 (DE3) is derived from the *E. coli* BL21 strain and is optimized for enhanced expression of eukaryotic proteins through the additional plasmid pRARE2, which supplies tRNAs for rare codons such as AGG, AGA, AUA, CUA, CCC, and GGA – codons frequently found in eukaryotic genes but uncommon in E. coli.

Induction was performed with 0.2 mM IPTG at +30°C, and after cells were grown to OD600=0.4 at +18°C. Two protein forms were expected: His-hSNM1B (~38.7 kDa) and SUMO/hSNM1B (~48.7kDa). Strong expression of a 38-40 kDa protein was detected in cells carrying pET28c/hSNM1B after 2.5 hours and overnight induction, whereas a strong band of 48-50 kDa was observed in cells containing pETHSUL/hSNM1B under the same conditions. The absence of the target protein before IPTG induction (0 hours) confirmed IPTG-dependent tight regulation of expression (Figure 1).



Note: Total protein samples were collected before induction (0h), after 2.5h, and following overnight induction with 0.2 mM IPTG. Lanes 1-3: E. coli expressing pET28c/hSNM1B; Lanes 4-6: E. coli expressing pETHSUL/hSNM1B. Mw markers: 14.4 kDa-116 kDa. Prominent bands at approximately 38-40 kDa and 48-50 kDa correspond to the expected His-hSNM1Bcut and SUMO-hSNM1Bcut fusion proteins, respectively. Molecular weight (Mw) indicators range from 14.4 to 116 kDa (Thermo Fisher Scientific PageRulerTM).

Figure 1. Expression of recombinant *hSNM1B* in *E. coli* Rosetta 2 (DE3) using pET28c and PETHSUL vectors

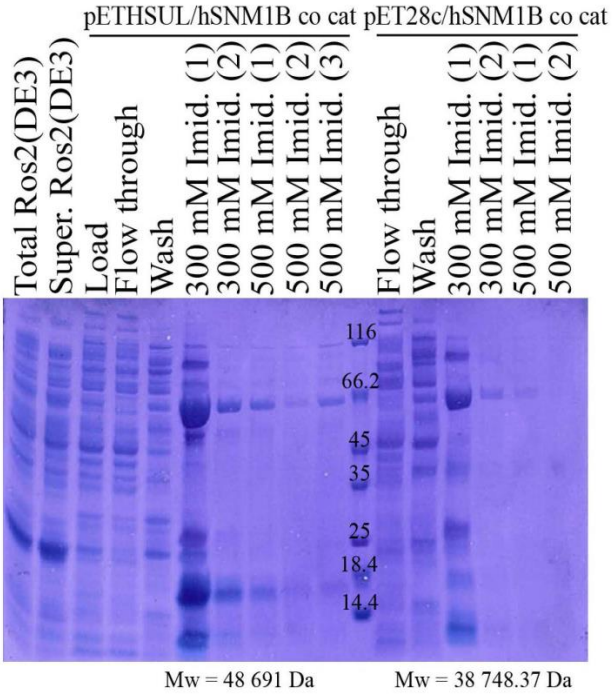
The expression of the recombinant *hSNM1B* protein in *E. coli* Rosetta 2 (DE3) was performed using the *pET28c/hSNM1B* and *pETHSUL/hSNM1B* vectors. Following IPTG induction, cell lysate samples were taken at three time points: before induction (0 hours), after 2.5 hours, and after overnight incubation (ON). SDS-PAGE was used to separate the proteins, and Coomassie staining was applied to visualize the protein bands in the gel. A band with a molecular mass of about 38.7 kDa, which corresponds to the hSNM1B protein with an N-terminal His6-tag, was detected in cell lysates that contained the *pET28c/hSNM1B* construct. A band of around 48.7 kDa, which represents the *hSNM1B* protein fused to the SUMO-tag, was been in bacterial cells containing

the *pETHSUL/hSNM1B* construct. The effectiveness of expression regulation is confirmed by the target band's appearance after and its absence prior to induction. The accumulation of recombinant protein is indicated by the band's increased intensity with extended induction. The presence of extra bands implies the need for additional, more extensive purification since they show concurrent proteins in the cell lysate (Figure 1).

This study compares the expression and purification efficiency of the human *hSNM1B* protein utilizing two distinct expression vectors, pET28c and pETHSUL in E. coli Rosetta2 (DE3) cells (Figure 2). SDS-PAGE analysis demonstrated that both constructs facilitated the successful overexpression of recombinant hSNM1B, exhibiting distinct bands at approximately 38.7 kDa for *pET28c/hSNM1B* and 48.7 kDa for *pETHSUL/hSNM1B*, aligning with the molecular weights of the His6-tagged and SUMO-fused proteins, respectively. Since most of the target protein was eluted at 500 mM imidazole, it was clear that the N-terminal His6-tag was strongly binding to the nickel affinity resin. Nevertheless, copurified contaminating proteins were also present in the elution fractions, indicating that a single affinity purification step is not enough to achieve high purity. According to other research, additional purification steps such as size-exclusion chromatography or a heparin affinity column may be required to obtain homogenous proteins with enzymatic activity towards DNA substrates. Remarkably, the SUMO-fusion construct *(pETHSUL/hSNM1B)* produced a more pronounced and distinct band as compared to that of the pET28c construct, indicating improved expression level and solubility. This conclusion is in line with earlier research that demonstrated that, in bacterial expression systems, SUMO fusion tags frequently enhance protein folding and solubility. The presence of a solubilizing fusion partner like SUMO probably helped to boost the amount of soluble protein because hSNM1B has several hydrophobic patches and predicted secondary structural components that can encourage aggregation (Figure 2).

Furthermore, the use of the Rosetta2 (DE3) strain, which provides tRNAs for rare codons, likely enhanced the production of this eukaryotic protein, consistent with prior findings on expression optimization in similar E. coli strains. Overall, the pETHSUL vector demonstrated superior solubility and expression yield, even though both constructs successfully enabled the recombinant *hSNM1B* protein production in E. coli. Future work will focus on additional purification and tag removal.

Next, we attempted to perform functional assays of the purified protein, such as measuring 5'→3' exonuclease activity toward fluorescently-labelled DNA duplex substrates. In vitro DNA exonuclease degradation assays were performed using Cy5-labelled DNA duplexes (cALi-1404-Cy5/G27 ApA) at 37°C for 30 minutes to assess the exonuclease activity of the recombinant *hSNM1B* protein. The substrates were incubated with varying concentrations of purified *hSNM1B*, and the reaction products were analyzed by denaturing Urea-PAGE followed by fluorescence detection (Figure 3). Lambda exonuclease (1-5U) (purchased from New England BioLabs) served as a positive control and, as shown in Figure 3, efficiently degraded the Cy5-labelled substrates, validating the reliability of assay conditions and the accessibility of DNA substrates to exonucleolytic 5'→3' degradation.



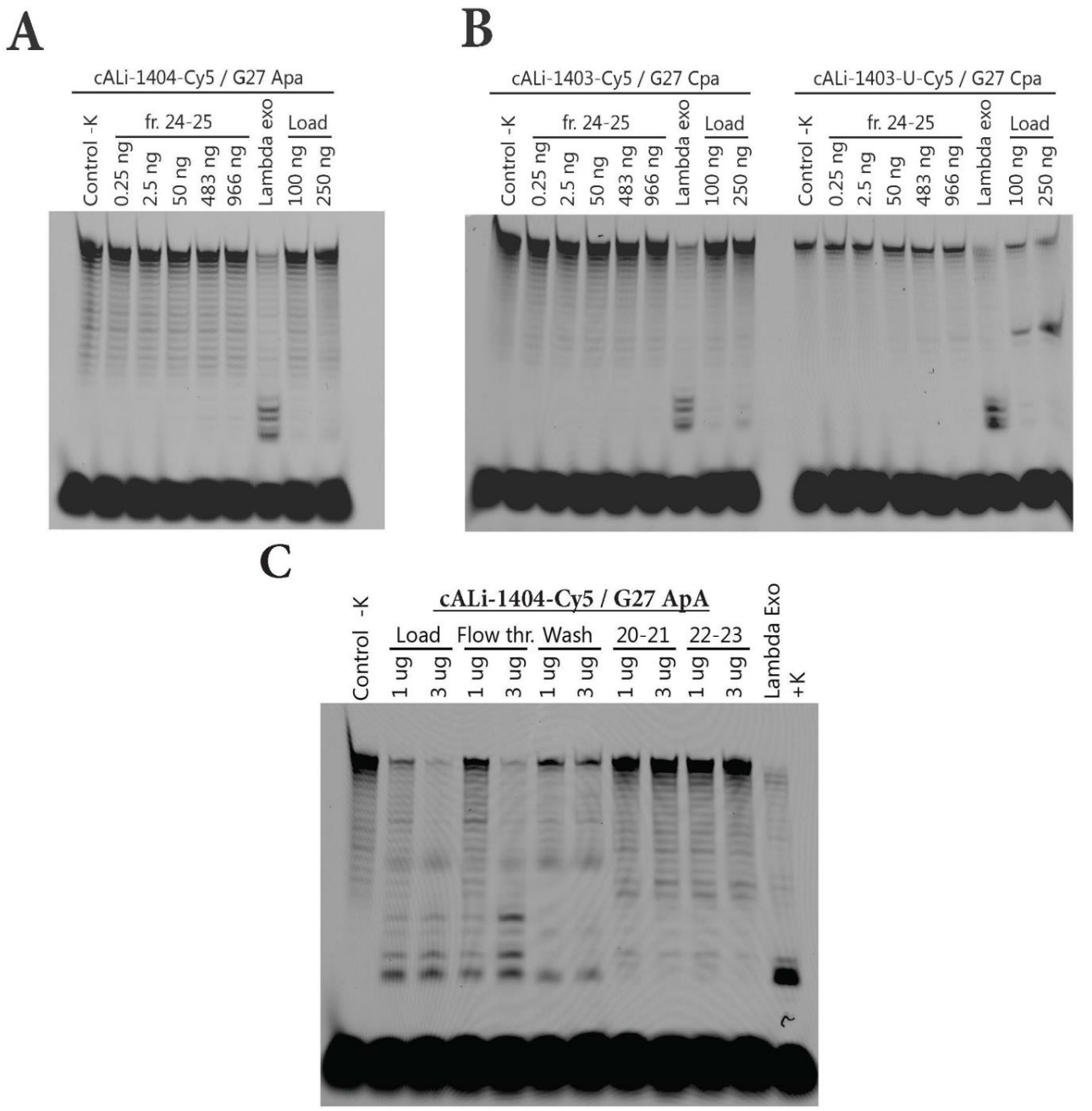
Note: Total lysate, soluble fraction, column load, flow-through, wash, and elution fractions were analyzed by SDS-PAGE. Samples correspond to E. coli Rosetta2 (DE3) cells expressing hSNM1Bcut from pETHSUL and pET28b vectors. Proteins were eluted with imidazole at concentrations of 300-500mM. Distinct bands of ~38.7 kDa (His-hSNM1Bcut) and ~48.7 kDa (SUMO-hSNM1Bcut) were observed in the elution fractions, indicating efficient expression and purification. Mw markers: 14.4-116 kDa (#26610, Thermo Fisher Scientific PageRulerTM).

Figure 2. Purification of recombinant *hSNM1B* proteins from *E. coli* Rosetta2 (DE3) using HisTrap HP columns

In contrast to λ nuclease, the samples incubated with the recombinant *hSNM1B* protein under the standard conditions did not exhibit any degradation of the labelled substrates. No lower-molecular-weight DNA fragments were observed, and the Cy5'-emitted fluorescence signal associated with the full-length substrate remained constant across all tested protein concentrations (2.5 ng to 250 ng). The negative control lanes, which lacked enzyme, confirmed that the substrates were stable and not subject to spontaneous or non-enzymatic degradation.

The presence of intact, full-length fluorescently labelled 27-mer DNA duplex in enzyme-free controls confirmed that any observed degradation would arise solely from enzymatic activity rather than from non-specific breakdown or contamination. As expected, the positive control (λ exonuclease) produced complete digestion of the DNA substrates, confirming the accuracy of the assay and its functionality.

Interestingly, the DNA substrates showed minor variations in degradation susceptibility. The cALi-1404-Cy5/G27 ApA substrate appeared more degraded at lower enzyme concentrations compared with cALi-1403-Cy5/G27 CpA and the modified cALi-1403-U-Cy5/G27 CpA substrate. These differences may reflect DNA sequence or structural variations (e.g., base modifications or secondary structures) that influence *hSNM1B*'s ability to initiate or extend 5′ → 3′ exonucleolytic cleavage. Specifically, the reduced degradation efficiency of the uracil-containing cALi-1403-U-Cy5 construct may indicate impaired recognition or processing efficiency.



Note: Lane labels indicate input load, flow-through, wash fractions, and positive control with λ-exonuclease. Negative controls (–K) confirm substrate stability in the absence of enzyme.

Figure 3. Exonuclease activity assay of the recombinant *hSNM1B* protein using 27-mer Cy5-labeled DNA duplexes. A) Cleavage of cALi-1404-Cy5/G27 ApA, B) cALi-1403-Cy5/G27 CpA, and cALi-1403-U-Cy5/G27 CpA duplexes incubated with increasing concentrations of purified *hSNM1B* (fractions 24–25). C) Cleavage of cALi-1404-Cy5/G27 ApA duplex incubated with increasing concentrations of purified *hSNM1B* (fractions 20–23)

Although the recombinant *hSNM1B* protein did not display measurable exonuclease activity under the current experimental conditions, the data confirm the production of soluble and

structurally intact protein, which represents a key result for further optimization. Future assays should explore alternative metal cofactors (e.g., Zn2+, Mg2+) modified buffer compositions, or DNA substrates that better mimic telomeric or cross-linked DNA structures known to be processed by SNM1B/Apollo.

Our results corroborate the solubility of purified *hSNM1B* proteins and validate fluorescently labeled synthetic DNA duplexes as substrates for 5'→3' DNA processing exonucleases. Also, the substrate-specific variations imply that, consistent with its documented role in DNA repair pathways, *hSNM1B* activity may depend on specific DNA sequence or base modification in the damaged duplex. Our knowledge of the substrate specificity and mechanism of *hSNM1B* will be improved by further biochemical investigations and comparison of the wild-type enzyme with its mutant variants.

Discussion

In this study, we optimized the expression and purification of the recombinant human nuclease Apollo (SNM1B), which plays a key role in telomere maintenance and DNA repair [15]. Our results showed that both the pet28c-*hSNM1B* and *pETHSUL/hSNM1B* constructs enabled robust protein expression in E. coli Rosetta2 (DE3) cells, albeit with significant variations in solubility and yield. Specifically, the SUMO-fusion construct (pETHSUL/hSNM1B) produced a stronger, more defined band (~48.7 kDa) on SDS-PAGE than the His6-tagged construct (~38.7 kDa), indicating improved folding and solubility (Figures 1 and 2). This finding is consistent with previous studies showing that SUMO tags often improve the solubility and stability of eukaryotic proteins in bacterial systems [31,32].

Although both expression vector constructs produce the recombinant protein, which binds efficiently to the Ni-NTA resin, the presence of co-purifying bacterial host proteins indicates that a single affinity step is insufficient to achieve high purity (Figure 2). This limitation has also been reported in studies of other SNM1-family nucleases, where additional purification steps such as size-exclusion chromatography or heparin affinity chromatography were required to obtain homogenous preparations suitable for biochemical analysis [7,9]. Thus, while we successfully established baseline purification conditions, further refinement will be essential for structural and biochemical investigations.

Functional assays using Cy5-labeled DNA duplexes revealed no detectable exonuclease activity of the recombinant *hSNM1B* protein under the conditions used (Figure 3). In contrast, Lambda exonuclease efficiently degraded the same DNA duplex, confirming the validity of the DNA substrate and assay. Several factors may explain this discrepancy. First, Apollo may require post-translational modifications or specific protein - protein interactions, such as those with TRF2 in the "shelterin" complex, to achieve its native activity [15,17]. Co-expression of these partners with Apollo or expression of the human nuclease in eukaryotic systems, such as Sf9 insect cells, HEK293 mammalian cells, or kidney-derived HKC8 epithelial cells, could restore the appropriate folding and post-translational modification states required for enzymatic activity. Second, the divalent cation dependency is a well-established feature of the SNM1 family of nucleases [8,11]. In the current assay, we used Mg2+ as the metal ion cofactor, but varying the type and concentration of metal cofactors (e.g., Mn2+, Co2+, Zn2+) or optimizing buffer conditions (pH, ionic strength, reducing agents) may enhance the catalytic performance of the Apollo enzyme. Third, substrate design could significantly influence the DNA nuclease activity. Although the Cy5-labeled duplexes contained CpA and ApA dinucleotide contexts, they may not fully replicate the physiologically relevant DNA substrates of SNM1B. Testing more complex substrates – such as

G-rich telomeric sequences, gapped or cross-linked DNA, or structures containing abasic sites – could provide better insight into the DNA substrate preference of Apollo.

Notably, DNA substrate-specific variations were observed, with ApA-containing duplexes showing relatively higher susceptibility to degradation compared to CpA or uracil-modified substrates (Figure 3). This observation supports prior reports that SNM1 nucleases exhibit sequence- and damaged base-context dependent activity [6,12].

In summary, this work establishes a strong experimental procedure for the production of the soluble human recombinant *SNM1B* protein in *E. coli,* with the SUMO-fusion strategy providing clear advantages in solubility and yield. Although enzymatic activity was not detected, the results reveal crucial experimental details that must be improved further - namely, expression host, metal cofactor composition, and DNA substrate design. Future work employing the eukaryotic expression system, or co-expression of shelterin components, coupled to systematic divalent cation testing, will be instrumental for reconstituting the active SNM1B protein to advance biochemical and structural studies. These studies will ultimately help to explore the SNM1B-catalyzed activities as potential targets for cancer therapy [6,23].

Conclusion

In summary, although the human recombinant *hSNM1B* protein did not display detectable enzymatic activity under the conditions used, a significant achievement of this study was the successful optimization of its expression and purification as a soluble protein from E. coli. Overcoming the common challenge of producing soluble human proteins in bacterial systems represents an important step toward further biochemical and structural characterization of DNA repair activities of *hSNM1B*.

The lack of observable activity may result from the absence of post-translational modifications, the use of non-specific DNA substrates, or the requirement for specific cofactors or interacting partners. Nevertheless, the established protocol for efficient expression and purification provides a robust foundation for subsequent biochemical studies aimed at refining reaction conditions, exploring eukaryotic expression systems, and testing physiologically relevant DNA substrates. Together, these efforts will facilitate a deeper understanding of the catalytic properties and biological role of *hSNM1B*, as well as its potential role in genome stability and cancer biology.

Author Contributions

A.A.A., A.M.T., U.B.S., K.O.S., M.K.S., and **S.M.T.** – conceptualization; **A.A.A., A.M.T.,** and **U.B.S.** – scientific design development; **A.A.A., A.M.T., U.B.S., K.O.S., M.K.S.,** and **S.M.T.** – implementation of the declared scientific research.

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Conflicts of Interest

Authors declare no conflicts of interest.

Compliance with ethical standards

This article does not contain a description of studies performed by the authors involving people or using animals as objects.

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Оптимизация экспрессии и очистки рекомбинантного белка Apollo *(SNM1B)* с использованием аффинной хроматографии

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Аннотация. Apollo (также известный как *SNM1B*) – нуклеаза, участвующая в стабилизации теломер и репарации ДНК, и его дисфункция связана с развитием ряда патологий, в том числе почечно-клеточной карциномы. Внимание к этому белку усилилось в связи с его потенциальной функцией в качестве мишени для противоопухолевой терапии. Настоящее исследование посвящено оптимизации экспрессии и очистки рекомбинантного белка Apollo (SNM1B) WT с помощью аффинной хроматографии. Для производства белка были использованы экспрессионные конструкции *pET28c-hSNM1BcoCut* и *pETHSUL-hSNM1BcoCut,* трансформированные в штаммы *Escherichia coli (E. coli)* Rosetta2 (DE3) и NovaXG. Вариации параметров индукции, таких, как концентрация IPTG, температура инкубации, присутствие детергента NP-40, глицерина, мочевины и продолжительность инкубации после индукции, были подвергнуты тщательному тестированию с целью определения оптимальных условий для получения растворимых рекомбинантных белков. Процесс очистки проводился с использованием аффинной хроматографии Ni-NTA. Чистота и растворимость белка оценивались с помощью SDS-PAGE. Полученные данные позволили определить эффективные условия для получения функционального и высокоочищенного белка Apollo, пригодного для дальнейших биохимических исследований, включая скрининг ингибиторов для онкотерапии.

Ключевые слова: репарация ДНК, Apollo, *SNM1B,* почечно-клеточная карцинома, повреждение ДНК, белок TRF2

Apollo *(SNM1B)* рекомбинантты белогының экспрессиясын және тазалығын аффинді хроматография әдісімен оңтайландыру

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Аңдатпа. Apollo *(SNM1B* деп те аталады) – теломерлердің тұрақтылығын қамтамасыз етуде және ДНҚ-ны жөндеуде қатысатын нуклеаза. Бұл белоктың функциясының бұзылуы бірқатар патологиялардың, соның ішінде бүйрек жасушалық карциноманың дамуына байланысты.

Бұл белокқа деген қызығушылық ісікке қарсы терапияның нысанасы ретіндегі потенциалды функциясына байланысты артты. Осы зерттеу рекомбинантты Apollo *(SNM1B)* WT белогының экспрессиясы мен тазалығын аффинді хроматография әдісі арқылы оңтайландыруға бағытталған. Белоктың өндірісі үшін *pET28c-hSNM1BcoCu*t және *pETHSUL-hSNM1BcoCut* экспрессиялық конструкциялары *Escherichia coli (E. coli)* Rosetta2 (DE3) және NovaXG штаммдарына трансформацияланды. IPTG концентрациясы, инкубация температурасы, NP-40 детергентінің, глицерин мен мочевинаның болуы және индукциядан кейінгі инкубация ұзақтығы сияқты параметрлердің өзгерістері еритін рекомбинантты белокты алу үшін оңтайлы жағдайларды анықтау мақсатында жүйелі түрде зерттелді. Тазалау процесі Ni-NTA аффиндік хроматографиясын қолдану арқылы жүргізілді. Белоктың тазалығы мен ерігіштігі SDS-PAGE әдісі арқылы бағаланды. Алынған мәліметтер функционалды және жоғары тазартылған Apollo белогын алу үшін тиімді шарттарды анықтауға мүмкіндік берді, бұл келесі биохимиялық зерттеулерге, соның ішінде онкотерапия үшін ингибиторларды скринингтеуге мүмкіндік береді.

Түйін сөздер: ДНҚ жөндеу, Apollo, *SNM1B*, бүйрек жасушалық карцинома, ДНҚ зақымы, TRF2 белогы

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