Indian J. Applied & Pure Bio. Vol. 41(2), 827-838 (2026). **ISSN: 0970-2091**

Expression, Purification and DNA binding activity of recombinant human XRCC1: A DNA repair gene

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Abstract

The XRCC1 (X-ray repair cross-complementing protein 1) is a DNA repair protein involved in repairing of damaged DNA. It primarily repairs DNA damage caused by alkylation, ionizing radiation, and the genotoxic agents. It acts as a molecular scaffold protein associated with single-strand DNA break repair and base excision repair pathway proteins like, poly nucleotide kinase, DNA polymerase, poly (ADP- ribose) polymerase I, and DNA ligase III, etc. The XRCC1 Open Reading Frame (ORF) encodes a 633-amino-acid protein with a 69.6 kDa molecular mass. Recently, a central DNA Binding domain is identified in the XRCC1 protein, whose complete characterization is not completely done. Therefore, the aim of this study is to characterize the XRCC1 protein Central DNA Binding Domain by studying its DNA binding activity by the DNA agarose gel based EMSA (Electrophoretic Mobility Shift Assay) technique, which is essential for interaction at damage repair sites. The pET16BHX recombinant plasmid vector construct was used to obtain enough recombinant XRCC1 protein expressing the N-terminus histidine deca histidine tagged recombinant protein in *E. coli*/BL21 cells after transformation. The recombinant XRCC1 protein was expressed in *E. coli*/BL21 cells as an N-terminus histidine-tagged protein of 69.6 kDa. We further confirmed it by 10% SDS-PAGE followed by western blot and observed ~100 kDa recombinant protein. This mobility difference was due to the presence of low numbers of aromatic, whereas high numbers of proline and acidic amino acids in the recombinant protein. We further purified the recombinant XRCC1 protein by Ni-NTA affinity column chromatography followed by western blot. Then we tested the functionality of the recombinant protein; we did the DNA agarose gel-based EMSA technique with nicked DNA and found that the recombinant protein is interacting with nicked DNA, and not with non- nicked DNA, which we further confirmed by the super shift assay. Thus, the Central DNA Binding Domain (CDBD) is important for mediating single-strand

DNA break repairs and base excision repairing of the DNA. This protein is absolutely required for maintaining genomic integrity of the cell. This study is the first step towards the analysis and characterization of the DNA-binding domain of XRCC1 protein *in vitro*.

Key words : Recombinant XRCC1 proteins; Protein Expression. *Escherichia coli*/BL21 cells; Neurological degeneration disease.

The human XRCC1(X-raycross-complementinggroup1) gene codes for a DNA repair protein in mammalian cells. It binds with the single strand break DNA, which is damaged by alkylated agents (e.g. ethyl methyl sulfonate). It also helps in the Base Excision Repair (BER) of DNA[14]. The XRCC1 protein is of 633 amino acids long peptide, which has 69.6 kDa Mol wt. The XRCC1 protein comprises of NTD (N-Terminal Domain), which is extended from 1 to 160 residue of amino acids. At C-Terminus of this Protein, it has BRCT1 & BRCT2 domains. Recently, Mac C. Y. M. *et. al.*, group has reported central DNA Binding domain (CDBD), which is extended from 219 aa to 415 amino acids[6]. By this domain XRCC1 interacts with damaged DNA (SSBD & BER DNA). It acts as a scaffold protein on which different proteins bind (like Lig3α, PNKP etc.) to do DNA repair and maintain genome integrity[4]. Thus, CDBD is crucial for the function of this molecule. Figure 1 is showing structure, different domains and interacting partners of XRCC1 protein[4,6].

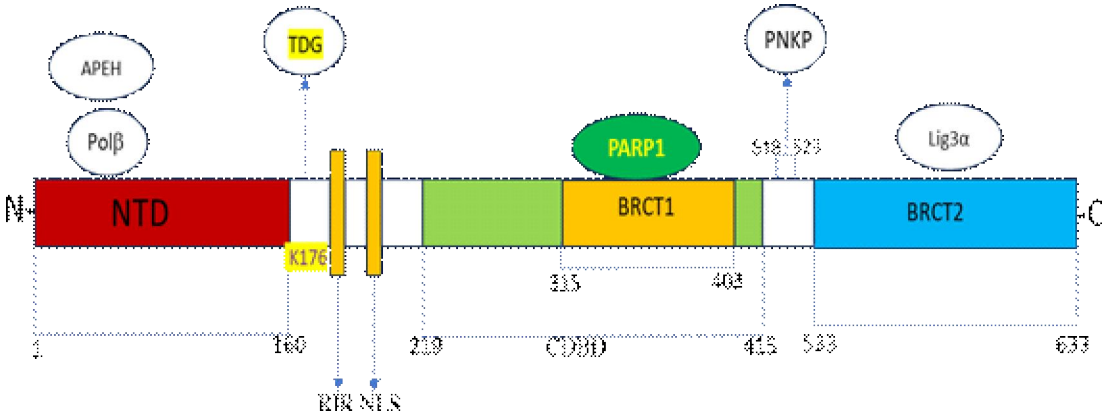

Figure 1. Structure of XRCC1 protein and its interacting proteins partner. (Modified from K. W. Caldecott 2018 & Mac C. Y. Mok, *et.al.*, 2019) NTD: N-terminal Domain, K: Lysine, RIR: Rev1-interacting region, NLS: Nucleus Localization Sequence, CDBD: Central DNA Binding Domain, BRCT1: BRCA1 C-terminus, BRCT2: BRCA2 C-terminus, APEH: Acyl peptide hydrolase, Pol_{β}: DNA $Polymerase_{\beta}$, TDG: Thymine DNA Glycosylase, PARP1: Poly (ADP-ribose) Polymerase 1, PNKP: polynucleotide kinase/phosphatase, Lig3α : DNA ligase 3α. Mathematical numbers are depicting numbers of amino acid residue.

In this study, we have expressed the recombinant XRCC1 protein in *E. coli*/BL21 cells from pET16BHX recombinant plasmid that harbors XRCC1 gene open reading frame (ORF). Then, we purified the recombinant XRCC1 from *E. coli*/BL21 cells supernatant lysate followed by a DNA agarose gel-based EMSA technique to study its DNA binding activity, which is important for its function. Our study is the first attempt to study this protein's DNA binding activity in *vitro,* which will allow finding the intrinsic property of this molecule.

Chemicals and Reagents :

Escherichia coli/BL21cells (Zymo Research, USA) were commercially purchased, pET16BHX (gifted by Prof. Keith W. Caldecott), All molecular biology grade reagents were obtained from HiMedia & Ni-NTA histidine resin (QIAGEN) used for histidine tagged protein purification. Poly-Prep Chromatography Columns Bio-Rad (Gravity column). Rabbit XRCC1 Antibody–BSA Free (NBP1-87154, Novus Biologicals) was kindly gifted by Prof. K.W. Caldecott.

Preparation of competent cells E. coli/BL21:

Day1: The *E. coli* cells used to prepare competent cells with Hanahan technique[7], the *E. coli* strain was rendered competent. It was then streaked on an LB agar (High Media: M575) plate with freshly prepared antibiotic (25mg/ml) and incubated at 37°C incubator overnight.

Day 2: Next day, single colony was selected to inoculate with the antibiotic 25 mg/ml in 5 ml of LB (High Media: M575), and incubated at 37°C at 200 rpm for overnight.

Day 3: From over-night culture 1% inoculums were used to inoculate in100 ml of LB (High Media; M575). The culture was incubated for about 2 hours at 37°C in shaking incubator at 200 rpm till growth reaches at an O.D.600nm ranging between 0.4-0.6. When culture reaches at required O.D.600nm then culture was immediately kept in ice and swirl to down the temp from 37°C, this culture was distributed in 50 ml of falcon tubes and centrifuged at 5000 rpm for 5 minutes at 4°C in Thermo Fisher Scientific X 2000 refrigerated centrifuge. After discarded the supernatant, pellets were mixed with 24 ml of MgCl2 (0.1M) (Titan Biotech; CAS No. 7786-30) and incubated at 4°C in ice for 20 minutes. This was followed by centrifugation of the cells at 5000 rpm for 5 minutes at 4°C in ice. Again, the supernatant was discarded and 12 ml of CaCl2 (0.1M) (Titan Biotech; CASNo.10035-04-8) was added and dissolved properly the cell pellet with a sterile Pasteur pipette. When no cell clumps were visible, the mixture was incubated at 4°C for 20 minutes on ice. This was followed by centrifugation of the cells at 5000 rpm for 5 minutes at 4°C.The supernatant was discarded and the cell's pellets were resuspended in 4.3 ml of $CaCl_2$ (0.1M) (Titan Biotech;10035-04-8) and 700 µl of 100 % glycerol (HiMedia; MB060). Then, aliquoted aliquots of 100 µl in each pre-chilled Micro Centrifuge Tube (MCT) were stored at -80°C to defreeze.

pET16BHX plasmid DNA transformation in competent cells of E. coli/BL21 :

The competent *E. coli* cells were thawed on ice at -80°C for 30 minutes and plasmid construct (10-20 ng) was added to ice-cold competent cells and chilled in ice for 30

minutes. Keep tapping regularly at an interval of 5 minutes. Heat shock was given by subjecting the cells to 42°C for exactly 90 seconds. After giving heat shock, it was kept in ice for 15 minutes. 400 µl of LB (HighMedia:M575) broth was Added and incubated for 90 minutes at 37°C. After incubation, 150 ml of pellet was spread on ampicillin (0.1 mg/ml) containing LB agar plate.

Expression of recombinant XRCC1 Protein in E. coli/BL21cells :

The human XRCC1 ORF construct pET16BHX plasmid DNA was obtained from K. W. Caldecott (Genome Damage and Stability Centre, UK). Following the technique of Caldecott *et.al*.,[2]; obtain protein XRCC1-His was expressed in *E. coli*/BL21 (Zymo Research, USA). It was transformed by the pET16BHX construct, which carries a histidine (deca times) tag at the N-terminus of human XRCC1 recombinant protein. Transformed cells were grown at 37 °C to an OD600nm of 0.6 in 20 mL of LB medium (High Media; M575) containing ampicillin at a concentration of 100 µg/ml. The pelleted cells were used to inoculate into 1L of LB media and then grown at 37°C to an OD600nm of 0.6. XRCC1 expression was induced at 37°C for 2 hours by addition of isopropyl 1-thio-â-D-galactopyranoside (IPTG) (G Bioscience; RC-063) to a final concentration of 1 mM. After that the cells were harvested by centrifugation at 8000 rpm at 4°C for 10 minutes, the cells were re-suspended in 20 ml of ice-cold sonication buffer (50 mM HEPES-NaOH, pH 8.0, 0.5 M NaCl, 0.1 mM EDTA, 10% glycerol), quick frozen in liquid nitrogen, and thawed on ice followed by addition of imidazole, (HiMedia; GRM559) dithiothreitol (HiMedia; MB070) and phenylmethanesulfonylfluoride SIGMA; P7626) to a final concentration of 1mM for each reagent. The bacterial cells were disrupted by sonication on ice, (2x30 seconds amplitude with 30 seconds cooling intervals on ice) and cellular debris removed by centrifugation (10000 rpm, 20 min, 4°C). The soluble fraction supernatant was obtained by centrifugation at 10000 rpm for 20 minutes. Supernatant collected after centrifugation and taken in 20 ml dilute in 5x loading dye (SDS 20%, 0.5M tris pH-6.8; Bromophenol Blue 5mg; Glycerol 100%; â- mercaptoethanol) and kept in boil in water for 5 minutes after spin sample at 10000 rpm for 5 minutes at room temp. Then load sample on 10% SDS- PAGE at 90v for 4 hours. Then gel was stained with commasiae brilliant blue R250.

Western Blot for detection of expression of recombinant XRCC1 protein :

Western blotting was used to confirm the expression of recombinant XRCC1 protein. For confirmation of expressed protein after induction of 1mM IPTG (G Bioscience; RC-063) and PAGE ruler plus protein marker 10-250 kDa were run on 10% SDS-PAGE (Tris1MpH8.8, Acrylamide 30% SDS 100 mg/ml, APS100 mg/ml) and transferred on to a PVDF (polyvinylidene fluoride) transfer membrane; Thermo Fisher Scientific by using Trans-Blot Turbo (Bio Rad). After the completion of electroblotting, 3% BSA (Titan biotech limited; CAS9048-46-8) were used to block PVDF membrane. Then after, it was kept for overnight at 4°C. After that, membrane was thrice washed the next day with TBST (Tris 1M, NaCl 150 mM, tween 20%) buffer (1xTBS,0.5%tween-20) for 10 minutes. Blot was incubated for 1 hour at RT on a rocker.

With Rabbit anti-XRCC1 polyclonal antibody (NBP1-87154) dilution (1:1000) in 3% BSA. Unbounded antibodies were removed with the 3-times washing with TBST (Tris 1M, NaCl 150 mM, tween 20 %) solution after a 10-minute interval at room temperature (RT). After washing, the membrane was incubated with secondary anti-XRCC1 antibody (Goat Anti-Rabbit, Ig GHRP conjugated) with 3% BSA dilution in TBST solution on rocker with room temperature (RT) for 2 hours. After 2 hours of secondary antibody incubation again, washing was done 3 times with TBST (Tris-Cl 1M, NaCl 150 mM, tween 20 %) was done followed by DAB staining to develop a membrane.

Purification of recombinant His-tagged XRCC1 protein :

The affinity of the His tag XRCC1 protein toward the Ni-NTA depends only on its primary structure. His–tagged proteins can be purified under native or denaturing conditions. While many recombinant proteins can be produced in a soluble form in *E. coli*, due to the relatively high affinity and specificity of the His-tag, a single IMAC (Immobilized Metal Affinity Chromatography) purification step in most cases of the target protein preparation is sufficient for many applications. However, in some cases, optimization of the purification process is required, especially if poorly expressed proteins are to be purified. Preparation of a cleared *E. coli* lysate under native conditions a cleared lysate from an *E. coli* cell pellet was generated for the purification of histidine-tagged proteins under native conditions. First, cell lysis was carried out using lysozyme, since it was inexpensive and very efficient for cells that had been frozen (Lysis methods based on physical disruption). The *E. coli* cells pellets were thawed on ice, resuspended in the cell pellet completely in 10 ml of lysis basis buffer (NaH_2PO_4 50 mM, NaCl 300 mM, Imidazole10 mM) pH-8, supplemented with 1mg/ml lysozyme, incubated for 30 minutes on ice, and centrifuged for 30 minutes at 10000 rpm at 4^o C. The supernatant (containing the soluble proteins) was collected. Then, 2.5 ml lysis buffer was added and gently resuspended the slurry to equilibrate the resin, allowing the resin to settle by gravity; then 2 ml of the supernatant was removed. 10 ml cleared lysate was prepared in step 1 to the equilibrate IMAC resin and incubated at 4ºC for 1-hour, rotating end–over–end. Flow through was collected after pouring the supernatant in the Ni-NTA packed column, followed by washing with a 5 ml wash buffer containing 40 mM and 80 mM Imidazole. The elution was carried out by containing 300 mM, 400 mM, and 500 mM imidazole.

DNase I treatment of gel purified IFN-VRE$_\beta$ DNA to create nick in substrate DNA :

To create nick in IFN-VRE$_\beta$ DNA using DNase I (Thermoscintific), we treated DNA for different time points, like 2 minutes, 4 minutes, 8 minutes, and 10 minutes in separate reactions to check timing and nick creation in substrate DNA. After DNase I treatment of VRE$_\beta$DNA with 37ºC temperature at different time points, like 8 minutes and 10 minutes, DNA was found to be degraded after 4 minutes of DNase I treatment incubation. Hence, only two minutes DNase I treated was used as DNA substrate for the EMSA experiment.

Electrophoretic mobility shift assay of recombinant XRCC1 protein :

For study of Protein–DNA interaction, the reaction mixture contained DNA substrate 150 ng in binding buffer (10 mM Tris pH 8, 100 Mm $NaCl_2$, 3mM $MgCl_2$, 1mMEDTA, 0.1%CHAPS, 10 mM, 2-marcato-ethanol, 7.5% Glycerol). The mobility shift assay with different concentration of purified recombinant XRCC1 protein (0.5 mg, 1 mg, 2 mg, 3 mg, 4 mg, 5 mg) was used and incubate at 37ºC for 30 minutes. The DNA–Protein complex was resolved in 1% agarose gel at 3 volt/cm for about 1-2 hours at 4ºC in 0.5 x TBE buffer. The chemi-Doc^{TM}MPimaging system was used for visualization of gel after ethidium bromide (0.5mg/ml) stanning for 1 hour.

Results

Expressionofrecombinant XRCC1

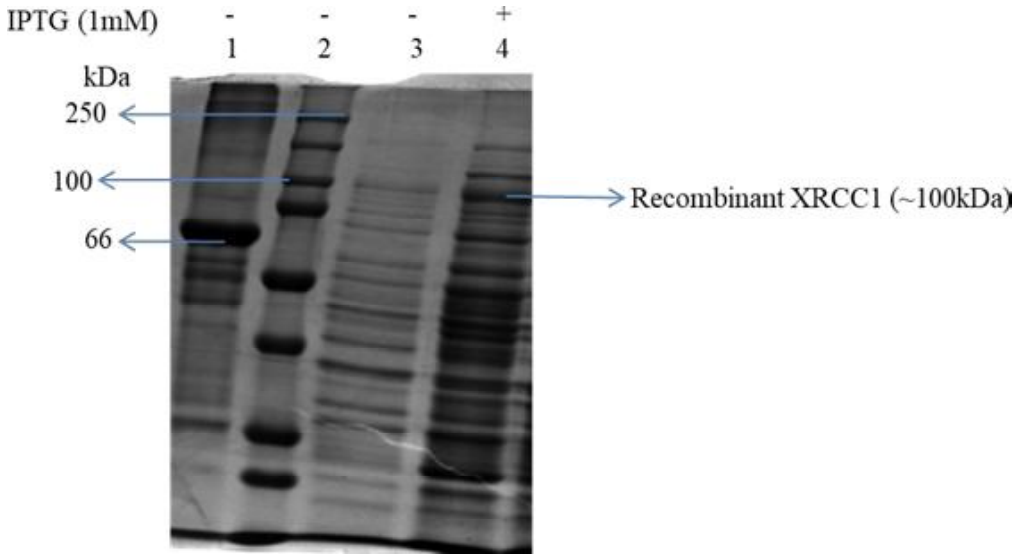

Figure 2. Expression of recombinant human XRCC1. 10% SDS PAGE is showing expression of recombinant deca-Histagged XRCC1 protein (Human), Lane1: BSA(~66kDa), Lane2: Protein ladder Marker (10-250kDa), Lane3: IPTG-Uninduced *E. coli*/BL21cells protein profiling, Lane4: IPTG-induced *E. coli*/BL21 cells showing expression of recombinant XRCC1 protein of ~100 kDa.

We successfully expressed recombinant XRCC1 in *E. coli*/BL21cells following transformation with the pET16BHX plasmid. Profound expression of recombinant XRCC1 was observed, as shown in Figure 2. The figure 2 presents protein profiling of *E. coli*/BL21 cells harboring the recombinant pET16BHX plasmid DNA. The recombinant protein includes an N-terminal deca-histidine (10 × His) tag. In Figure 1: Lane1 shows bovine serum albumin (BSA) loaded as a control of~66 kDa molecular mass, Lane 2 displays the Protein ladder of molecular mass range from (10- 250 kDa) Lane 3 demonstrates no

expression XRCC1 recombinant protein due to absence IPTG in culture and Lane 4 is showing expression of recombinant XRCC1 of ~100 kDa mass following induction with 1 mM IPTG for 3 hours at 37 °C.

Western blot of recombinant XRCC1

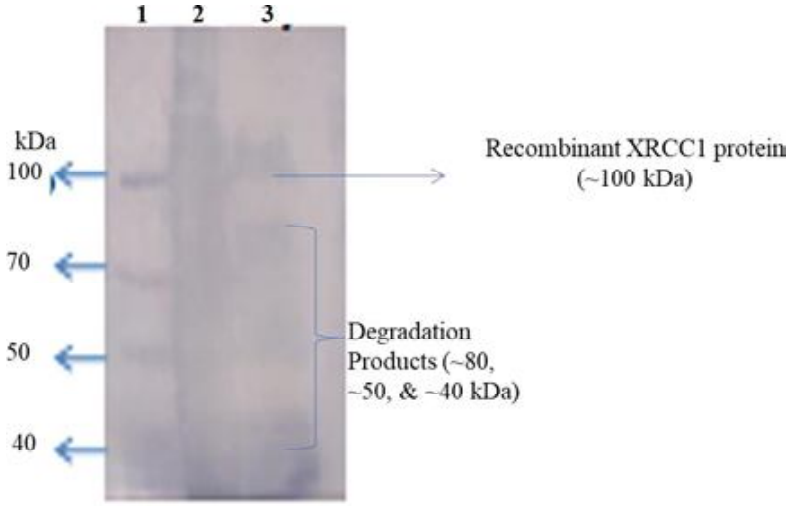

Figure 3. Western blot for detecting the expressed recombinant XRCC1 protein using anti XRCC1antibody. Lane 1: Protein Marker (40-100 kDa), Lane 2: Total cell lysate, Lane 3: Induced Supernatant showing recombinant XRCC1 protein band along with a few degradation products.

To further confirm that the expressed recombinant protein was indeed XRCC1, we performed a western blot using an anti-XRCC1 antibody. Figure 3 shows; Lane1 Protein ladder (10–250 kDa). Lane 2 shows total cell lysate from IPTG-uninduced culture, which as expected shows no detectable band. Lane 3 shows total cell lysate from IPTG-induced culture, displaying a prominent band at ~100 kDa, corresponding to the full-length XRCC1 recombinant protein. In addition, several lower molecular weight bands (~80, ~50, and ~40 kDa) are also observed. These low molecular mass bands likely represent degradation products of the full-length XRCC1 protein. In *E. coli*, recombinant proteins are often recognized as foreign and subjected to proteolytic degradation, resulting in truncated fragments. To minimize such degradation, expression at reduced temperatures (*e.g.*, 30°C, 24°C, or 16°C) can be employed, as lower temperatures generally decrease protease activity and improve protein stability.

Effect of XRCC1 protein on the growth of E. coli/BL21 cells :

We observed (see figure 4) no any toxic effect of XRCC1protein on *E. coli* cells as OD at 600nm wavelength it is increasing with time. With IPTG induced clone not showing any toxic effect to *E. coli* cells. See Table-1.

Table-1.

XRCC1clone (UN & IND)	OD_{600nm} at 0 hour	$OD_{600\ nm}$ at 15minutes	$OD_{600\ nm}$ at 30minutes	$OD_{600\ nm}$ at 45minutes	$OD_{600\ nm}$ at 60minutes	$OD_{600\ nm}$ at 120minutes
UN	0.052	o.079	0.105	0.135	0.159	0.240
IND	0.062	0.090	0.119	0.146	0.180	0.304

Thus, XRCC1 protein is not toxic to *E. coli*/BL21 cells.

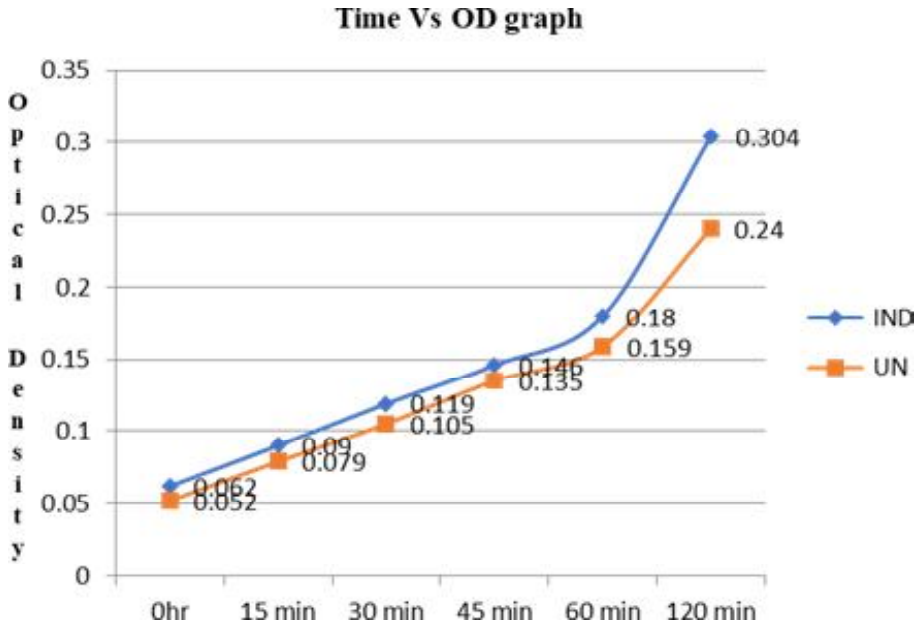

Figure 4. Time Vs O.D. of growth curve of recombinant XRCC1 expressing in *E. coli* cells. clone at 0 hour, 15 minutes, 30 minutes, 45 minutes, 60 minutes, 120 minutes after induction by 1mM IPTG.

Purification of recombinant XRCC1 protein:

After identifying the expressed recombinant protein, we purified recombinant XRCC1 using Ni- NTA column affinity chromatography. Figure 5 shows protein profiling by 10 % SDS-PAGE. Lane 1 contains BSA (~66 kDa) as a standard control. Lane 2 (Sup.) shows the IPTG-induced supernatant, indicating expression of recombinant XRCC1 along with additional low molecular weight bands (~80, ~50, and ~40kDa). Lane 3 (TCL) represents the total cell lysate. Lane 4 shows the flow-through, while lanes 5 and 6 correspond to the wash fractions. Lanes 7–10 (E1-E4) display the elution with 300 mM, 400 mM, 500 mM and 600 mM imidazole, revealing the purified recombinant protein along with a few lower molecular weight bands. These smaller bands are likely degradation products of the full-length protein.

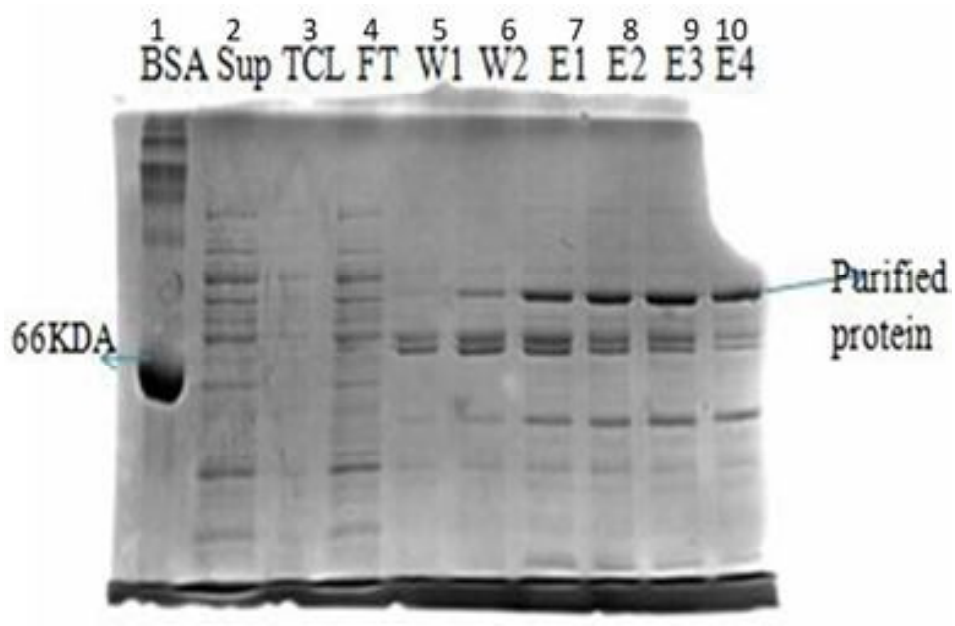

Figure 5. Purification of recombinant XRCC1 protein. Lane1: BSA(~66kDa), Lane 2: Supernatant,1ane 3: total cell lysate, lane 4: Flow through, Lane 5: washing 1 was done by 40 mM imidazole, lane 6: Wash 2 by 80 mM imidazole, lane 7: Elution1was carried out by 200 mM imidazole, lane 8: Elution 2, 300 mM imidazole, lane 9 Elution 3 by 400 mM imidazole, lane10 Elution 4 by 500mM imidazole.

EMSA study of recombinant XRCC1 protein with nicked DNA substrate :

For the EMSA study, we used VRE_{β} DNA substrate (Virus Response Element of the IFN_{β} gene), which is 346 bp in length. Nicked DNA was generated by DNase I treatment for 2 minutes at 37°C, and the DNA binding activity of recombinant XRCC1 was then assessed using this substrate. Figure 6 shows the agarose gel EMSA of recombinant XRCC1 with nicked VRE_{β}DNA. In lane 1, no binding was observed when recombinant XRCC1 was incubated with the non-nicked DNA substrate, serving as a negative control. In contrast, when nicked VRE_{β}DNA was used, a single band shift was detected with 5 mg purified recombinant XRCC1 protein, indicating that recombinant XRCC1 is functionally active and specifically binds to nicked DNA (lane 2). In lane 3, incubation with 5.5µg of purified XRCC1 protein resulted in a clear-cut band shift, that confirmed the nicked DNA binding activity. In lane 4, an additional shift was observed when XRCC1 antibody was included in the reaction. This enhanced shift, known as a super-shift, demonstrates the formation of a complex between XRCC1, the antibody, and the nicked DNA substrate. Together, these results confirm that recombinant XRCC1 interacts specifically with nicked DNA, but not with intact non-nicked DNA.

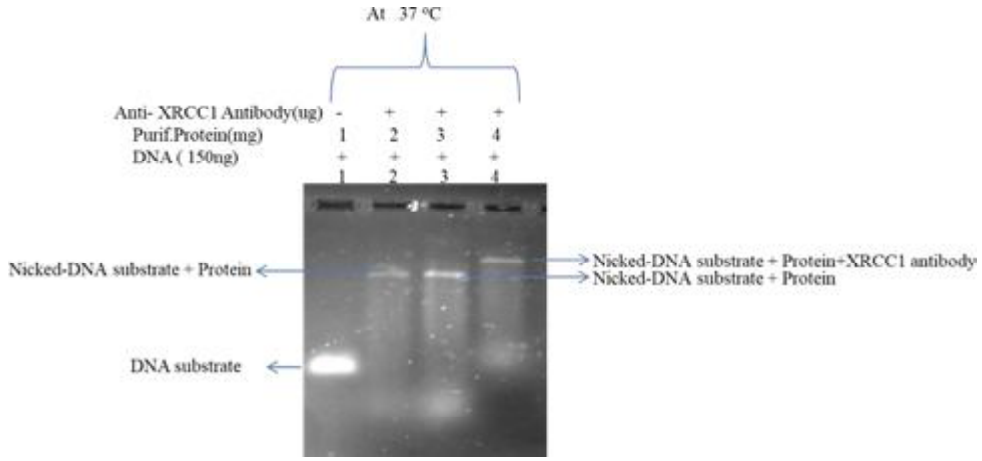

Figure 6. DNA agarose gel based EMSA: 2% TBE agarose gel showing EMSA study using purified XRCC1 protein. DNA binding activity of recombinant of XRCC1 protein on VRE (Virus Response Element) of IFNb gene was used as a DNA substrate. Lane1: Non-nicked DNA substrate with purified XRCC1 protein, Lane 2: EMSA with nicked DNA substrate with 5 mg purified Protein, Lane 3: EMSA with 5.5 mg Purified protein and lane 4: Super-shift assay with anti XRCC1 antibody at 37ºC.

The XRCC1 protein is a scaffold protein that mediates the assembly of DNA repair factors at sites of single-strand breaks (SSBs). Such breaks arise not only from environmental genotoxic stress but also during normal metabolic processes, where torsional strain generated during DNA replication is relieved by topoisomerases[5]. If unrepaired, SSBs can lead to genetic disorders such as cerebellar ataxia and oculomotor apraxia—complex neurodegenerative diseases of which no cure currently exists[1]. Repair of SSBs is initiated when they are sensed by PARP (poly [ADP-ribose] polymerase I [1] (PARP-1) interacts with the BRCT1(315 to 403 amino acids) domain of XRCC1, facilitating recruitment of repair proteins[9]. Recent studies have shown that the BRCT1 domain lies within the central DNA-binding domain 219 to 415 (amino acids) (CDBD) of XRCC1. This domain is therefore essential not only for SSB repair but also for the epigenetic regulation of gene expression through base excision repair (BER). Our research focuses on the central DNA-binding domain of XRCC1, as the CDBD is the primary interface for interactions with both SSB and BER DNA substrates.

The expression of the recombinant XRCC1 protein from the pET16BHX plasmid is shown in Figure 2. The expressed recombinant protein showed anomalous electrophoretic mobility of ~100 kDa in SDS-PAGE as it is of ~70 kDa protein. In different literature, it is reported that it is moving around ~85 kDa in SDS-PAGE[10] and in few literatures it has been shown that it is moving around ~100 kDa protein size[8]. The major anomalous electrophoretic mobility difference is due to the presence of a low number of aromatic amino

acids and a higher number of proline and acidic amino acids. The higher content of negative charged acidic amino acids in the protein makes it unable to interact with SDS to human XRCC1 protein, resulting in deviation from normal movement in SDS-PAGE Figure 3 shows~100 kDa protein full-length human XRCC1 expression along with a fewer low molecular mass proteins band of ~80, ~50, and~40 kDa. These bands are degradation products of full- length XRCC1 protein. This result agrees with the result of Mok M. C. et. al., 2019), in which he proved that N-terminal deca-histidine tag showed full-length human XRCC1 protein expression along with fewer degraded XRCC1 protein bands, whereas C-terminal deca-histidine tagged XRCC1 could not show any degradation products.

We report in this research article that the XRCC1 protein exhibits no cytotoxic or toxic effects on the growth of *E. coli*/BL21 cells. Our observations indicate that the presence of XRCC1 does not influence the growth pattern of *E. coli* overtime. To the best of our knowledge, no previous studies have documented this finding. Therefore, this is the first exclusive report on the effect of XRCC1 protein on *E. coli* growth.

Purification of recombinant XRCC1 yielded the intact XRCC1 protein along with fewer numbers of degradation products. This result is consistent with the findings reported by K. W. Caldecott and colleagues[2] in their 1995 Nucleic Acids Research paper, where similar observations were made. Other studies have also noted that recombinant proteins expressed in *E. coli* are prone to degradation, as the bacterial proteolytic machinery recognizes them as foreign proteins and targets them accordingly[15].

The recombinant XRCC1 protein was functionally active, as it specifically interacted with nicked DNA substrates but not with non-nicked DNA. We further demonstrated that the recombinant protein was expressed in the soluble fraction of *E. coli* rather than in the insoluble fraction (data not shown). The purified XRCC1 protein bound to nicked DNA, and its identity was confirmed by a super- shift assay. In figure 6, Lane 1 shows the non-nicked DNA substrate, which did not interact with XRCC1, as no band shift was observed. In contrast, lanes 2 and 3 demonstrate that XRCC1 interacted exclusively with nicked DNA, resulting in band shifting. To confirm that the interacting protein was indeed XRCC1, we performed a super-shift assay using an anti-XRCC1 antibody, which produced an additional shift. These binding interactions are attributed to the central DNA-binding domain of XRCC1, spanning amino acids 219–415. Our findings are consistent with the report by Demin *et. al.*,[6] group, which highlighted the importance of this domain[6]. Thus, the central DNA-binding domain is crucial for the DNA repair function of XRCC1. This study represents the first step toward analyzing XRCC1 *in vitro* and provides a platform for future investigations aimed at elucidating the molecular mechanisms of XRCC1-mediated DNA repair *in vivo*.

The human XRCC1 recombinant protein was successfully expressed in *E. coli*/ BL21 cells transformed with the pET16BHX recombinant plasmid. The protein was purified, although a few lower molecular mass bands (~80, 50, and ~40 kDa) were also detected, which likely represent degradation products of

the full-length protein. Functional assays demonstrated that the recombinant XRCC1 protein specifically interacts with nicked DNA, but not with intact non-nicked DNA. This work represents an initial step toward characterizing the Central DNA Binding Domain (CDBD) of XRCC1 *in vitro*. Further studies are warranted to elucidate the intrinsic properties of the CDBD and its role in XRCC1 function.

Our heartfelt gratitude goes to Prof. K. W. Caldecott (Genome Damage and Stability Centre, UK) for generously providing the XRCC1construct and the anti-XRCC1 antibody. We are equally indebted to Dr. Vahab Ali (Scientist F, RMRI, Patna) for his valuable advice and for supplying essential chemicals and instruments that greatly supported my research. Financial support in the form of UGC fellowship to SZ is gratefully acknowledged.

Conflict of Interests :

There is no any conflict of interest.

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