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Subcloning, Expression and Purification of Functional E. coli Nucleotide Excision Repair Protein UvrA Using IMPACT-CN System

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by

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ABSTRACT

Subcloning, Expression and Purification of Functional *E. coli* Nucleotide Excision Repair Protein *Uvr*A Using IMPACT-CN System

by

Cathy Lin

DNA in cells is constantly damaged by both endogenous and exogenous genotoxic agents. Nucleotide excision repair (NER) in *Escherichia coli* (*E. coli*) is one of the DNA repair systems that recognizes and removes a variety of DNA damage such as pyrimidine dimers, bulky chemical adducts, DNA intrastrand cross-links, etc. The genes responsible for *E. coli* NER incisions are *Uvr*A, *Uvr*B, and *Uvr*C. Purification of *Uvr*A, *Uvr*B, and *Uvr*C is essential for research to understand the molecular mechanisms of NER and carcinogenesis. Although *Uvr*A has been successfully purified in our lab in the past, the experimental procedures were very time-consuming and technically challenging. In this study we employed IMPACT (Intein Mediated Purification with an Affinity Chitin-binding Tag) system to subclone the cDNA of *Uvr*A and express and purify the recombinant *Uvr*A protein by a single-column step. The purified *Uvr*A protein was found to be fully functional in the *Uvr*ABC incision assay.

DEDICATION

This research work is dedicated to my beloved and my family for their support, advice, and encouragement.

ACKNOWLEDGEMENTS

I would like to thank my graduate committee for their guidance and mentorship in my graduate study. Thank you Dr. Yue Zou for chairing my committee and for your advice. Through this experience I have gained invaluable knowledge under your help and support. I would want to thank Dr. Phillip Musich and Dr. Cerrone Foster for your advice, encouragement, knowledge, and assistance with my research. I have acquired valuable skills under the guidance of all of you and I will be able to attain my professional goals because of you.

I would also like to thank my fellow graduate students in our lab for their constant help with my research and friendship. Finally, I would like to thank my family and in particular my husband for the massive amount of support and energy they put forward to help me accomplish this degree.

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CHAPTER 1

INTRODUCTION

DNA in cells is under constant attack from both endogenous and exogenous genotoxic agents (Zou et al. 1995; Zou et al. 2004; Young et al. 2005; Liu 2007). Exogenous sources could be manmade mutagenic substances and naturally occurring agents such as sunlight and dietary mutagens. Reactive oxygen species formed during cellular metabolism could be one of the endogenous sources. According to the type of DNA structural alterations, the DNA damage can be classified as damage to the nucleotide base, damage to the phosphodiester backbone, and DNA cross-links. The results can lead to permanent changes in the genetic information encoded in the DNA due to its mutations. The DNA damages are physical and chemical abnormalities in the DNA molecule. The DNA repair of a cell is vital to the integrity of its genome and thus to the normal functionality of that organism. In response to this threat, cells detect and repair the DNA damage.

A number of DNA repair systems have evolved and each of them has developed to specialize in the repair of certain types of damage. Nucleotide excision repair (NER) is a major repair pathway that is known for removal of stretches of bases containing various lesions such as pyrimidine dimers, bulky chemical adducts, DNA intrastrand crosslinks, and some forms of oxidative damages. The common features of these lesions are DNA duplex helical distortion and DNA chemical modification (Zou et al. 1995; Zou et al. 2001; Zou et al. 2003; Yang et al. 2005; Zou et al. 2005). Soon after the first detection of NER in 1960 when the excision of UV-induced DNA lesions in bacteria was observed (Setlow and Carrier 1964; Boyce and Howard-Flanders

1964), following studies revealed the genes responsible for bacterial NER incisions are *Uvr*A, *Uvr*B, and *Uvr*C (Hanawalt and Haynes 1965).

The NER pathway in *Escherichia coli* also involves the *Uvr*ABC proteins. The *Uvr*A, *Uvr*B, and *Uvr*C proteins recognize and incise damaged DNA in a multistep reaction (Figure 2). In solution, *Uvr*A dimer formation is driven by ATP (Mazur and Grossman 1991). *Uvr*A forms either an *Uvr*A_2B (Orren and Sancar 1989) or *Uvr*A_2B_2 (Verhoeven et al. 2002) complex with *Uvr*B. *Uvr*A initiates the DNA contacts and transfers the DNA to the DNA binding domain of *Uvr*B (Della Vecchia et al. 2004). *Uvr*B is considered the central recognition protein in the bacterial NER system (Sancar et al. 1988; Orren et al. 1992), and its cryptic ATPase activated in the presence of the *Uvr*AB:DNA complex is necessary for damage verification. The interaction of *Uvr*A_2B with the damage causes unwinding, denaturing, and opening of the local DNA duplex at the adduct (Zou and Van Houten 1999). *Uvr*A hydrolyzes ATP, resulting in its self-dissociation from the recognition complex and leaving behind a *Uvr*B:DNA preincision complex (Orren and Sancar 1990). *Uvr*B must be in its ATPase-bound conformation (Moolenaar et al. 2000) before 3' incision by *Uvr*C, which is responsible for both the 3' and 5' incision reactions (Verhoeven et al. 2000). The *Uvr*BC complex is a structure-specific, ATP-dependent endonuclease (Zou et al. 1996). The first incision is at the fourth phosphodiester bond 3' of the lesion, and the second incision is at the eighth phosphodiester bond 5' of the damaged base (Sancar and Rupp 1983; Lin and Sancar 1992; Lin et al. 1992; Zou et al. 1995; Verhoeven et al. 2000). Following incision, *Uvr*C dissociates and DNA helicase II (*Uvr*D) is required to release the incised oligonucleotide containing the lesion (Sancar and Rupp 1983; Lin and Sancar 1992; Lin et al. 1992; Verhoeven et al. 2000). DNA polymerase I fills this gap and removes *Uvr*B from the nondamaged DNA stand (Caron et al. 1985; Hasain et al. 1985). DNA ligase I joins the newly synthesized end to the parental DNA, thus completing the NER pathway. Overall, *Uvr*A plays a vitally important role in the NER mechanism because it is the first component of the system to recognize DNA damage. The *Uvr*A gene encodes a 115 kDa protein. Sequence analysis has revealed the presence of 2 zinc fingers and 2 ATP-binding cassette ATPase domains. *Uvr*A (Figure 1) consists of 2 halves (white and yellow, respectively) separated by a flexible protease sensitive linker region.

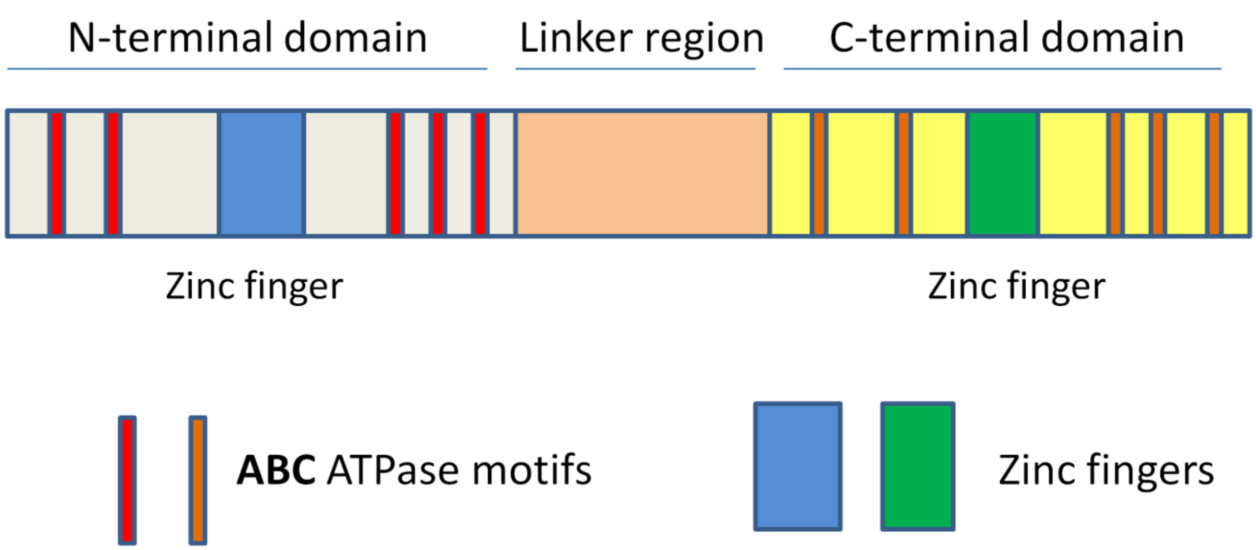

Figure 1: Linear representation of the gene *Uvr*A. Two domains are separated by a flexible protease-sensitive linker region. Within each domain, there is one ABC ATPase motif, a zinc finger. The 2 domains are shown in gray and yellow, while the linker region is shown in beige. The conserved ABC ATPase motifs are shown in red and orange and the zinc fingers are shown in blue and green.

It is likely that the C-terminal zinc finger is primarily responsible for *Uvr*A's DNA binding capacity that facilitates the NER reaction (Houten et al. 2005; Truglio et al. 2006). N-terminal domain possessed both the ability to dimerize and hydrolyze ATP. The dimerization of *Uvr*A is in a head-to-head fashion (Myles and Sancar 1991), and it could be a key regulatory point in the NER pathway. *Uvr*A is a DNA-independent ATPase that can hydrolyze both ATP and GTP (Seeberg and Steinum 1982; Caron and Grossman 1988; Truglio et al. 2004).

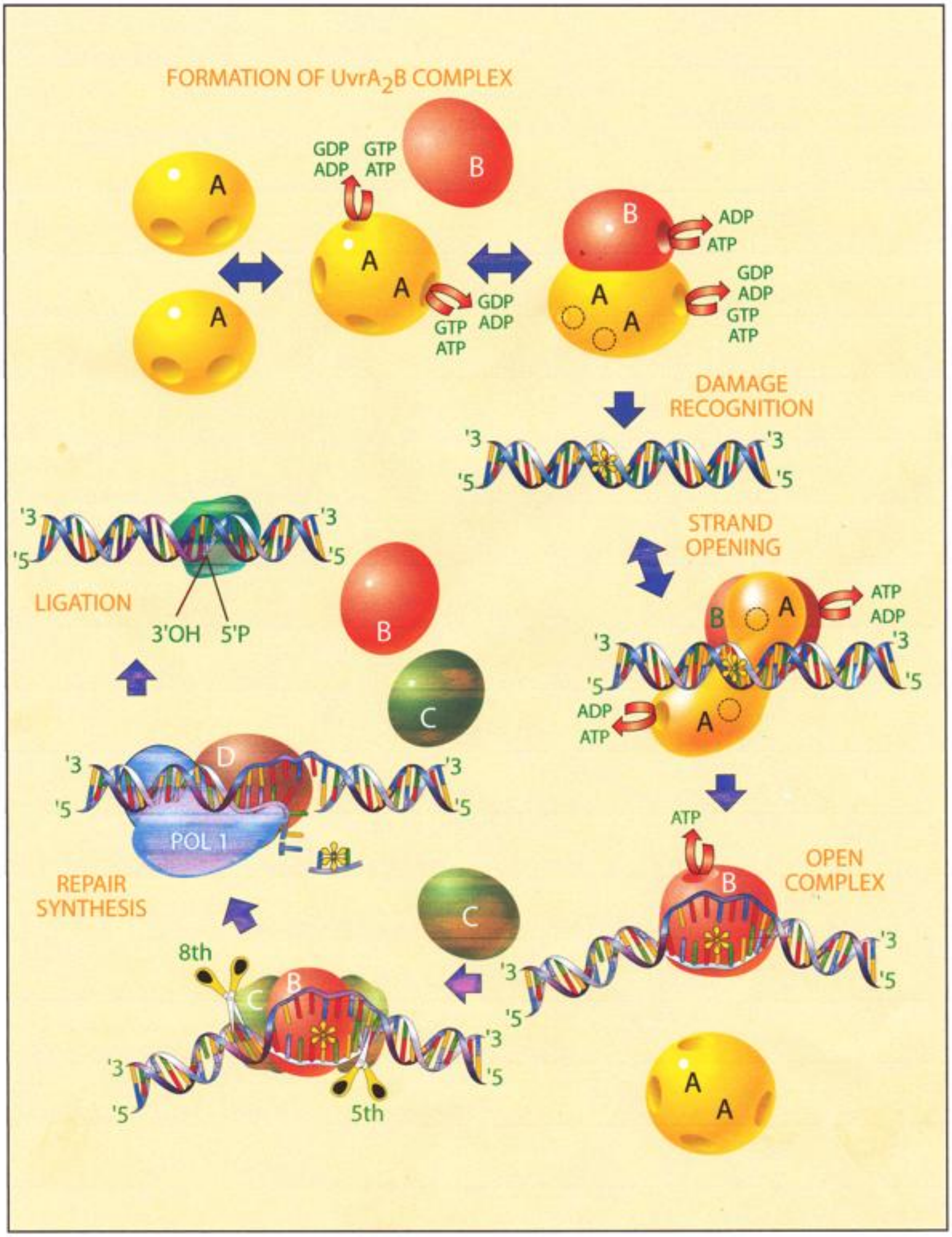

Figure 2: Prokaryotic nucleotide excision repair (Shell 2008)

Protein *Uvr*A, *Uvr*B, and *Uvr*C are required for the bacterial NER research study in our lab. Although the protein *Uvr*A has been successfully purified in the past, the experimental procedures were very time-consuming and technically challenging. In this study we employed IMPACT (Intein Mediated Purification with an Affinity Chitin-binding Tag) system, a single-column step method, to subclone the gene of *Uvr*A, and express and purify the recombinant *Uvr*A protein. The IMPACT-CN system (Figure 3) is an intein-based affinity protein purification system. Intein, encoded from the *Saccharomyces cerevisiae* VMA1 gene, is a protein splicing element with 454 amino acid residues. In this system there is an inducible (controllable) peptide bond cleavage reaction involved either at the N-terminus or C-terminus of an intein. To release the target protein from the fusion protein, the peptide bond cleavage is triggered or induced by addition of thiols such as dithiothreitol (DTT), β-mercaptoethanol or free cysteine at either N-terminus or C-terminus of an intein. Specific mutations at the C-terminal splice junction of the intein allow C-terminal peptide bond cleavage induced by addition of thiols such as DTT, resulting in elution of the target protein while leaving the intein tag remains on the affinity column (NE BioLabs Inc. manual Page 3).

The pTYB vectors contain a T7/*lac* promoter to provide strict control of the fusion gene expression. These vectors use their own copy of the *lac* I gene encoding the *lac* repressor. Binding of the *lac* repressor to the *lac* operator suppresses basal expression of the fusion gene in the absence of IPTG (isopropyl *β*-D-1-thiogalactopyranoside) induction. The pTYB vectors also carry the *bla* gene to encode ampicillin selective marker, which conveys ampicillin resistance to the host strain. The pTYB2 vector is one of the examples, and it uses a T7/*lac* promoter and carries its own copy of the *lac* I gene and *bla* gene (NE BioLabs Inc. manual Page 7).

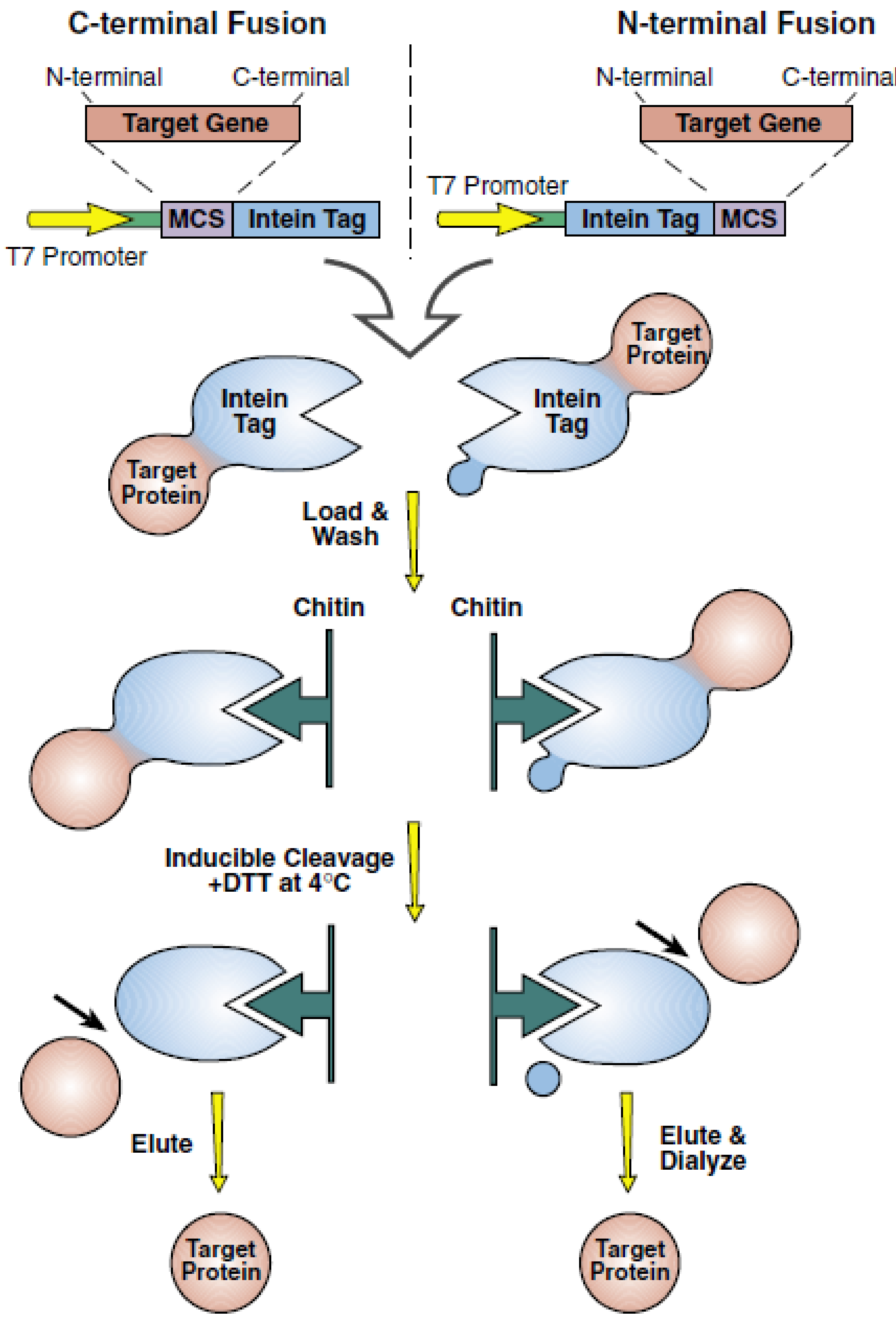

Figure 3: Schematic illustration of IMPACT system (NE BioLabs Inc. manual Page 3)

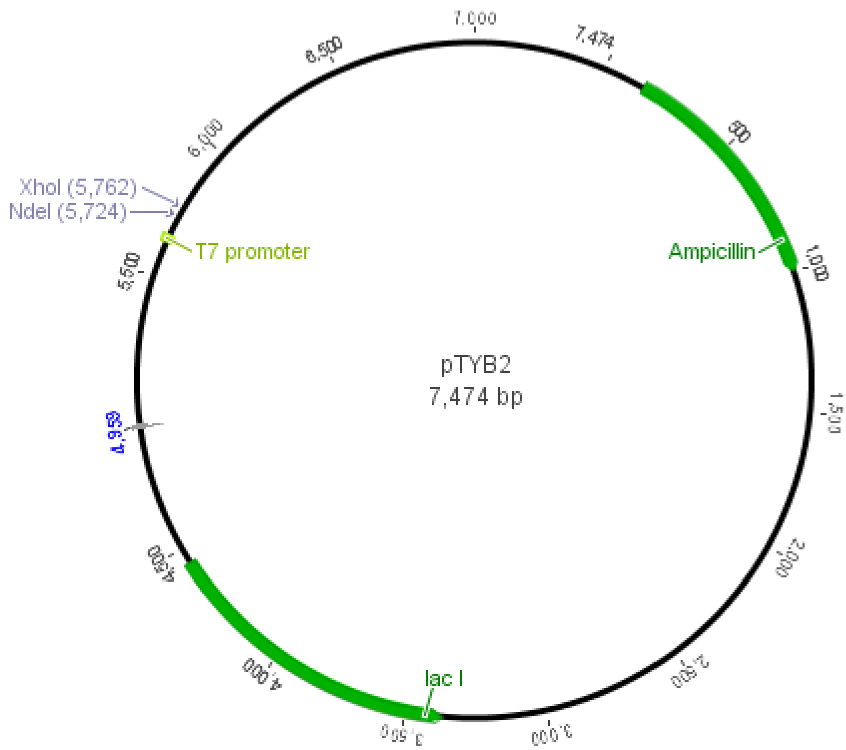

Figure 4: pTYB2 vector

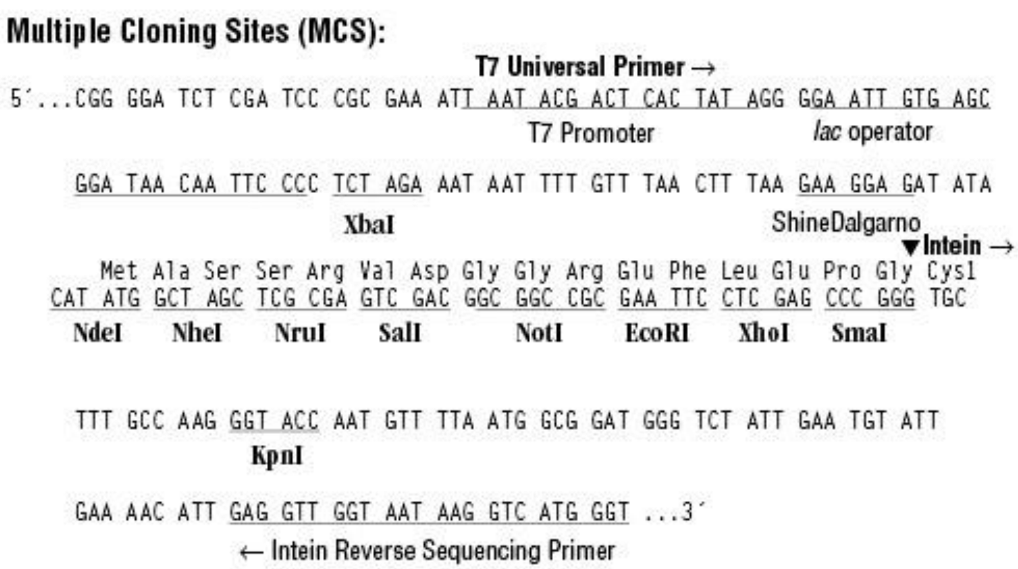

Figure 5: The multiple cloning sites of pTYB2 vector (NE BioLabs Inc. manual page 8)

In this project we chose the C-terminal fusion vector pTYB2 (7,474 bp; Figure 4), which allows the fusion of the cleavable intein tag to the C-terminus of the target protein *Uvr*A, for cloning and expression of recombinant protein *Uvr*A in *E. coli* cells. pTYB2 uses ATG of the Nde I site in the multiple cloning region (Figure 5) for translation initiation. Use of the Xho I site pTYB2 yields the target protein *Uvr*A with 3 (glycine, proline, and glutamate) extra residues at the C-terminus of target protein *Uvr*A after the cleavage of the self-cleavable intein tag. The chitin binding domain (CBD) in the intein tag allows the binding of the fusion precursor protein *Uvr*A_intein to a chitin column. The intein tag undergoes specific self-cleavage in the presence of thiols, such as DTT, *β*-mercaptoethanol, or free cysteine and releases the target protein *Uvr*A from itself resulting in a single-column purification of the target protein *Uvr*A.

CHAPTER 2

MATERIALS AND METHODS

Subcloning of *Uvr*A Gene into pTYB2 Expression Vector

For subcloning of the *Uvr*A gene from plasmid pSST10 carrying *Uvr*A (supplied by L. Grossman, Johns Hopkins University), two DNA oligodeoxyribonucleotides, 5' sense primer, containing a NdeI-restriction site GGG AAT TCC ATA TGA TGG ATA AGA TCG AAG TTC GGG, and 3' antisense primer, containing a XhoI restriction site CCG CTC GAG CAG CAT CGG CTT GAG GAA G were synthesized. The *Uvr*A gene was amplified by polymerase chain reaction (PCR) in a 50 microliter (µl)-reaction mixture containing 1 x LongAmp Taq reaction buffer (60 mM Tris-SO_4, 20 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 3% Glycerol, 0.06% IGEPAL® CA-630, 0.05% Tween® 20, pH 9 @ 25°C), 300 micromolar (µM) deoxynucleotide triphosphates (dNTPs), 400 picomolar (pmol) of each primer, 30 nanogram (ng) template DNA (pSST10_*Uvr*A) and 2 units (2 µl) LongAmp Taq DNA polymerase using following conditions: I cycle (initial denaturation): 94^0C for 30 seconds; 30 cycles: 94^0C for 30 seconds, 58^0C for 60 seconds, 65^0C for 3 minutes, followed by 65^0C for 10 minutes. The PCR product was purified using QIAquick PCR purification kit available from Qiagen (Cat. 28104). Purified PCR product as well as vector pTYB2 were double-digested with restriction endonucleases NdeI + XhoI, vector pTYB2 was dephosphorylated with alkaline phosphatase (1 unit of FastAP, Thermo Scientific, #EF0651). Both DNA fragments were purified from 1% agarose gel following gel electrophoresis and extracted by QIAquick Gel Extraction kit (Qiagen, cat.28704). Purified DNA fragments of *Uvr*A and vector pTYB2 were used (molar ratio 3:1) in ligation reaction by 1 unit of T4 DNA ligase (Promega, M1801) at 4^0C overnight.

Ligation mixture was transformed into *E. coli* DH5α competent cells. The plasmids from Amp^R transformants were isolated, analyzed by digestion with both Nde I and Xho I restriction endonucleases, and sequenced to confirm that both junctions' sequences between *Uvr*A DNA fragment and the pTYB2 vector were correct.

Overexpression of *Uvr*A Protein

The resulting recombinant plasmid DNA, pTYB2-*Uvr*A, was transformed into *E. coli* C41 (DE3) cell, a derivative of BL21 (DE3). The fresh Amp^R transformants were used to inoculate 10 ml lysogeny broth (LB) medium, with final concentration 100 µg/ml ampicillin, for 1 hour at 37^0C first, then this culture was transferred into 1 liter of LB medium (with 100 µg/ml ampicillin) and grew for 2.5 hours at 37^0C with shaking (200 rpm) until the optical density at 600 nm (OD_{600}) of the culture was around 0.6, then 0.7 mM IPTG was added. The IPTG induction of UvrA was at 30^0C for 6 hours with 200 rpm shaking. The culture was harvested by 6,000 rpm centrifugation and cell pellets were stored at -20^0C overnight.

Purification of *Uvr*A Protein

The pellets from 1 liter of culture were resuspended in 80 ml of column buffer (20 mM Tris-HCl, pH 8.0; 500 mM NaCl; 0.1 mM EDTA; 0.1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were lysed by 1 pass through French press at 8,000 psi, and clarified by centrifugation at 13,200 rpm for 30 minutes at 4^0C. The clarified extracts were used for loading the chitin column (10 ml) at a rate of no faster than 0.5 ml/min after chitin beads were equilibrated with 15 volumes of column buffer. The column then was washed with

30 column volumes of column buffer at 1 ml/min flow rate. For inducing the on-column cleavage reaction of the fusion protein *Uvr*A-intein, the column was flushed quickly with 3 column volumes of cleavage buffer [20 mM Tris-HCl, pH 8.0; 500 mM NaCl; 0.1 mM EDTA; freshly diluted 30 mM dithiothreitol (DTT)]. The flow in the column was stopped and the cleavage was allowed to continue at 4^0C overnight. Protein *Uvr*A was eluted using additional 3 column volumes of cleavage buffer without DTT, and was collected in 1 milliliter fractions for total of 20 milliliters. Fraction 2 and fraction 4 were examined using PAGE for molecular weight and purity of isolated protein. Samples from some major steps of overproduction and purification of *Uvr*A protein were separated on a 10% SDS-polyacrylamide gel that was stained with Coomassie blue and photographed. Such samples included that from cell extracts of noninduced cultures, IPTG-induced cell cultures, the flow-through of extract loading, column washing, and on-column cleavage induction, chitin resin, and elution fractions 2 and 4, respectively. Fractions 1-10 (pool-1) and 11-20 (pool-2) were pooled and dialyzed against one liter of storage buffer (50 mM Tris-HCl; 100 mM KCl; 0.1 mM EDTA; 0.1 mM DTT; 50% glycerol). The obtained *Uvr*A protein samples were aliquoted into several microcentrifuge tubes and stored at -20^0C until the biological activity was confirmed.

*Uvr*A Protein Concentration

The concentrations of *Uvr*A protein samples were determined by the microplate procedure (Thermo Scientific Pierce 660nm Protein Assay) with bovine serum albumin (BSA) as a standard. A standard curve was prepared within the assay's working range. 10µL of each replicate of standards [800 µg/mL, 400 µg/mL, 200 µg/mL, 100 µg/mL, and double-distilled (dd)-H_2O], *Uvr*A protein samples (1:10 dilution with dd-H_2O), and the blank samples (storage buffer in 1:10 dilution with dd-H_2O) were added into the microplate well of 96-well plates. Then

150 μL of the protein assay reagent was added into each well. The plate was covered and mixed on a plate shaker at medium speed for 1 minute, and incubated at room temperature for 5 minutes. The blank wells were used to zero the plate reader first, and the absorbance of the standards and *Uvr*A protein samples were measured at 660 nm. A standard curve was prepared by plotting the average blank-corrected 660 nm measurement for each BSA standard versus its concentration in μg/mL, and this standard curve was used to determine the concentrations of *Uvr*A protein samples from pool-1 and pool-2. The concentrations of pool-1 and pool-2 are 2.0 μM and 1.3 μM, respectively.

*Uvr*ABC Incision Assays

The 5' termini ^{32}P-labeled DNA substrates (4 nM) containing FABP [N-(20-deoxyguanosin-8-yl)-4-fluoro-4-aminobiphenyl] adduct were incised by *Uvr*ABC (*Uvr*A, 20 nM; *Uvr*B, 250 nM; UvrC, 100 nM) in *Uvr*ABC buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl2, 5 mM DTT, 1 mM ATP) at 37^0C for a specified length of time. *Uvr*ABC enzymes were diluted and premixed in *Uvr* storage buffer (20 mMTris-HCl, 500 mM NaCl, 1 mM EDTA, 50% glycerol) prior to addition to incision reaction mixes. Aliquots were collected at 0, 10, 20, and 30 minutes into the reaction. The reaction was terminated by heating at 95^0C for 5 minutes. The products were denatured into single strands by addition of formamide 6 x dye (bromophenol blue and xylene cyanol) loading buffer and heating to 95^0C for 5 minutes, and immediately plunged into ice to prevent reannealing. Digested (incision) products then were analyzed by gel electrophoresis on a 12% polyacrylamide sequencing gel under denaturing conditions with TBE buffer (50 mM Tris-borate, 1 mM EDTA pH 8.0). ^{32}P-labeled incision products or nonincision DNA bands in the gels were visualized using a Fuji FLA-5000 image scanner with MultiGauge V3.0 software.

CHAPTER 3

RESULTS

Obtaining *Uvr*A Gene from the Plasmid pSST10 Carrying UvrA

The *Uvr*A gene was amplified from the plasmid pSST10 carrying *Uvr*A by PCR (Polymerase Chain Reaction) before it could be cloned into the expression vector pTYB2 (7474 bp) using the IMPACT™ kit (NEB). The first step was the design of the forward (5' sense) and reverse (3' antisense) primers: forward primer containing a NdeI-restriction site GGG AAT TCC ATA TGA TGG ATA AGA TCG AAG TTC GGG, and reverse primer, containing a XhoI restriction site CCG CTC GAG CAG CAT CGG CTT GAG GAA G were synthesized. The PCR products were purified and double-digested with restriction enzymes NdeI & XhoI, and were analyzed by 1% agarose gel electrophoresis (*Uvr*A gene has 2,823 bp) as indicated in Figure 6.

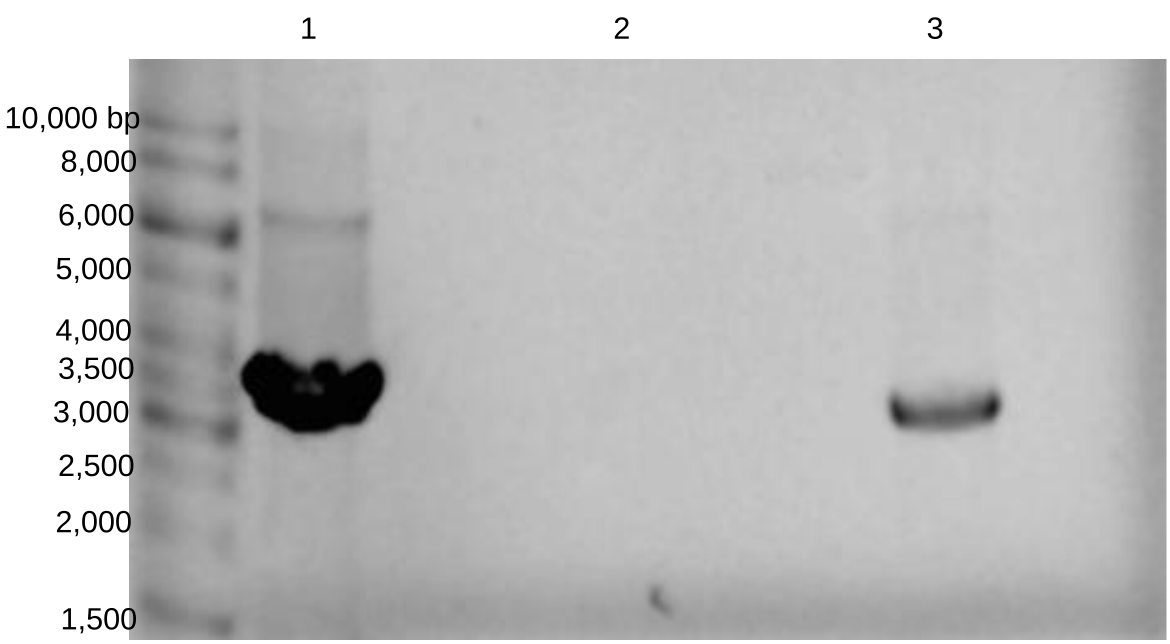

Figure 6: Analyzing the PCR Reaction Products of *Uvr*A Gene. Products of PCR reaction using pSST10_*Uvr*A plasmid as the template as the positive control (Lane 1). Products of PCR reaction using dd-H_2O as the template as the negative control (Lane 2). Purified PCR products were double-digested with restriction enzymes NdeI and XhoI (Lane 3).

Preparing the C-terminal Fusion Vector pTYB2 For *Uvr*A Gene Insertion

The vector pTYB2 purchased from New England Biolabs was first amplified by transformation into DH5α competent cells and plasmid isolation. The newly amplified plasmid of pTYB2 vector then was confirmed by the digestion with various restriction endonucleases and analyzed by 1% agarose gel electrophoresis (Figure 7). Meanwhile, the efficiency of restriction endonucleases Xho I and Nde I was affirmed.

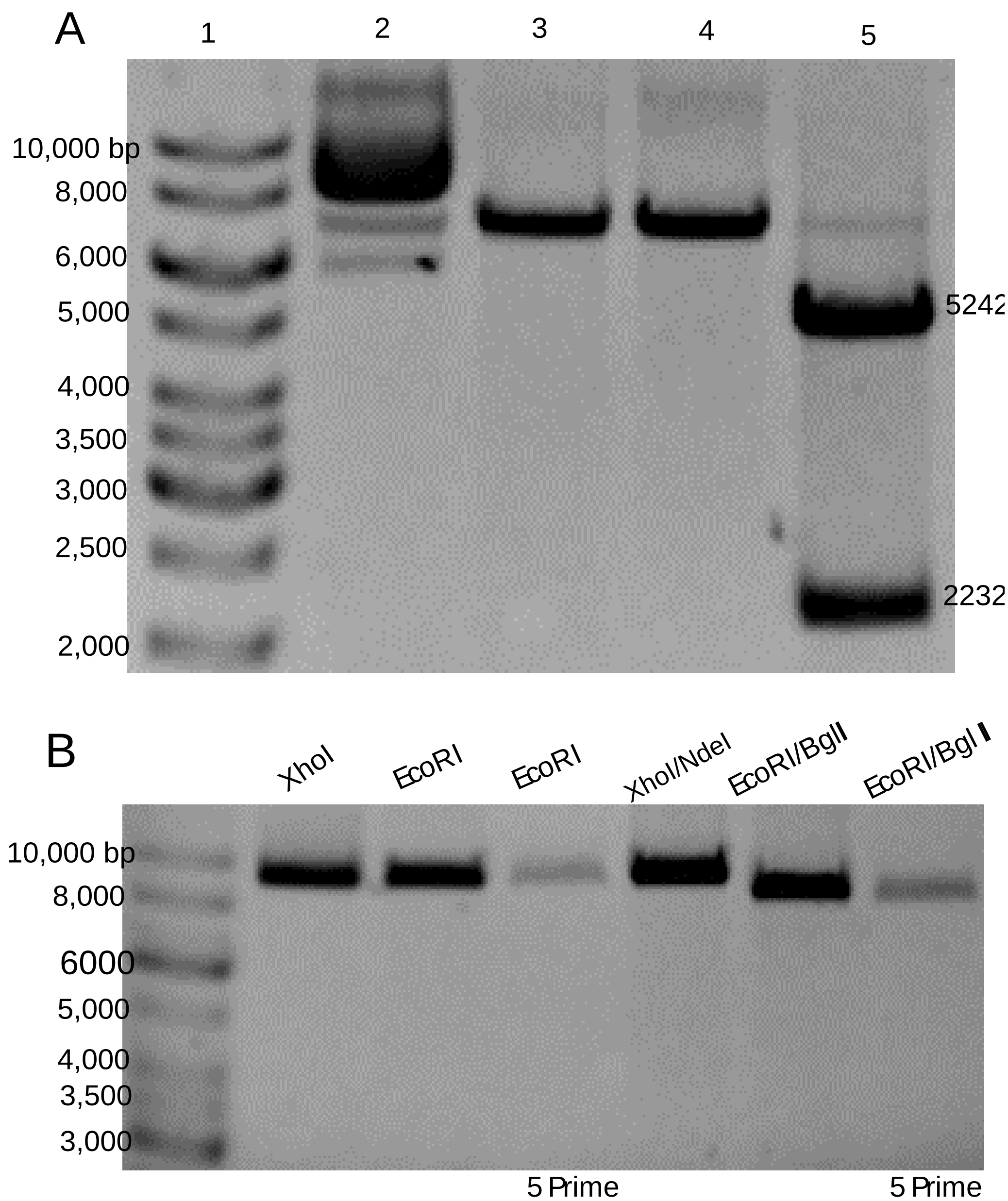

Figure 7: Confirmation of amplified pTYB2 with various restriction endonucleases. (A) DNA Marker (GeneRuler 1 kb DNA Ladder, Thermo Scientific #SM0313) (Lane 1). Circular pTYB2 plasmid (non-cutter) (Lane 2). pTYB2 digested with restriction endonuclease Xho I (single cutter) (Lane 3). pTYB2 digested with restriction endonuclease Nde I (single cutter) (Lane 4). pTYB2 digested with restriction endonuclease Bgl I (dual cutter) (Lane 5). (B) pTYB2 digested with single cutters Xho I, EcoR I, Xho I/Nde I, EcoR I/Bgl I, respectively. QIAprep Spin Miniprep kit (Cat. 27104) were used for the plasmid isolation and purification except 2 samples were purified by PerfectPure DNA Kit (by 5 Prime, Cat. 2900289) as indicated in the figure.

Ligation and Transformation

Purified PCR products as well as vector pTYB2 were double-digested with restriction endonucleases Nde I and Xho I. The vector pTYB2 was further dephosphorylated with alkaline phosphatase (FastAP). Both DNA fragments were purified from 1% agarose gel electrophoresis (Figure 8) using QIAquick Gel Extraction kit. The bands showing in lane 1 and 2 are double-digested vector pTYB2 and *Uvr*A, respectively.

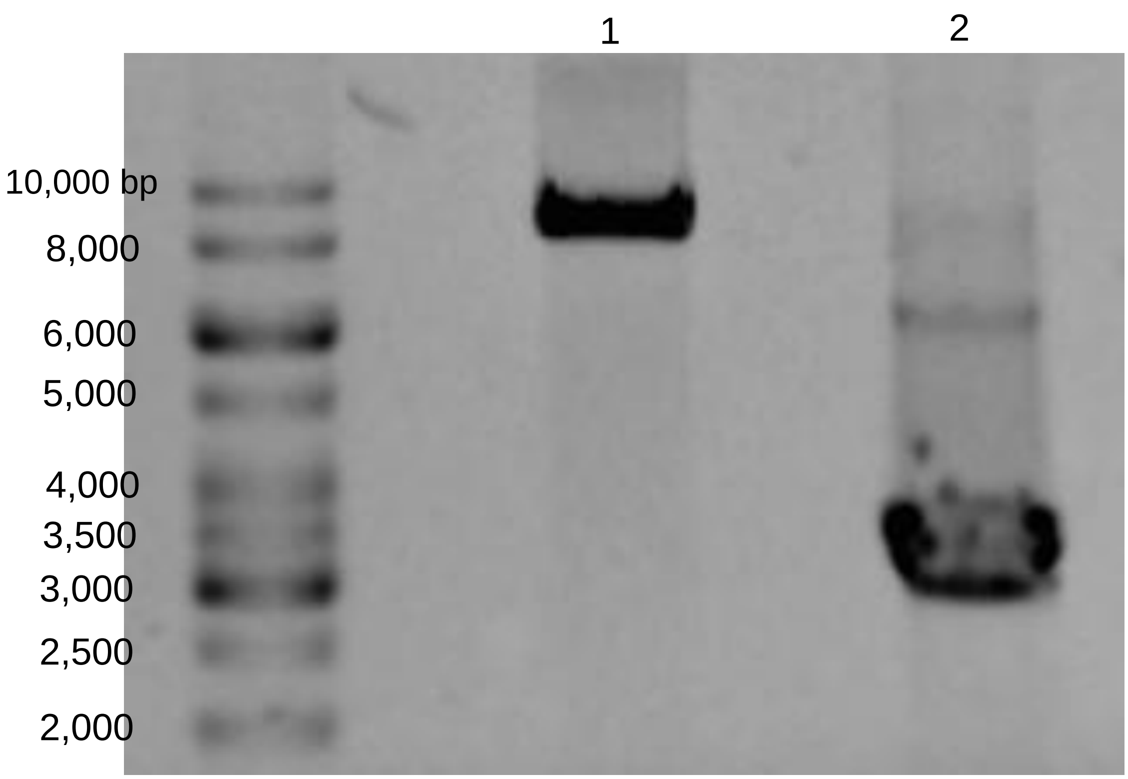

Figure 8: The purification of *Uvr*A and vector pTYB2 fragments for the ligation. Vector pTYB2 was double-digested with restriction endonucleases NdeI and XhoI, and then dephosphorylated with FastAP (alkaline phosphatase) (Lane 1). PCR products (*Uvr*A fragment) were double-digested with restriction endonucleases NdeI and XhoI (Lane 2). Both of DNA fragments were purified from 1% agarose gel electrophoresis using QIAquick Gel Extraction kit.

The *Uvr*A DNA fragment and the prepared vector pTYB2 were combined into a reaction with the enzyme T4 DNA ligase at 4^0C overnight, which covalently links free ends of DNA together. This ligation reaction mixture was transformed into *E. coli* DH5α competent cells under heat shock, which allows putting ligated pTYB2_*Uvr*A DNA plasmid into the bacterium. The transformants then were plated onto LB-ampicillin agar media, which allowed only cells that had successfully taken up ligated pTYB2_*Uvr*A DNA plasmid to grow. After the incubation at 37^0C overnight, there were over 20 colonies found growing on the LB-ampicillin agar media plate.

Plasmid Isolation from Amp^R Transformants

Isolated plasmids named 1 through 6, and A, B, and C were obtained from 2 different ligation reactions and transformations, respectively. The purified plasmids from all 9 of these colonies were used as the templates for the PCR reactions by the pair of primers for *Uvr*A gene described in Experimental Procedures, and the PCR products were analyzed by 1% agarose gel (Figure 9). Very likely, all these plasmids isolated, including colony B, harbored *Uvr*A gene (2,823 bp).

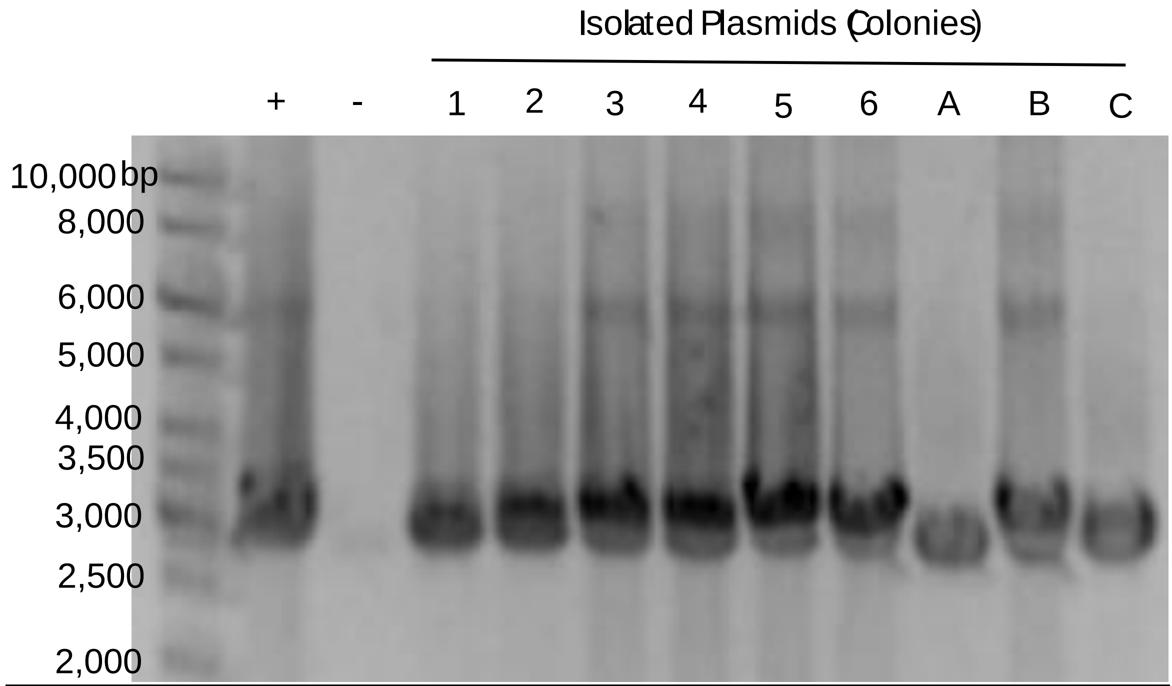

Figure 9: PCR Products using isolated plasmids as the templates. PCR products using pSST10_*Uvr*A plasmid as the template (positive control). PCR products using pcDNA3.1(+)_ATR plasmid as the template (negative control). Isolated plasmids named 1 through 6, and A, B, and C were obtained from 2 different ligation reactions and transformations, respectively.

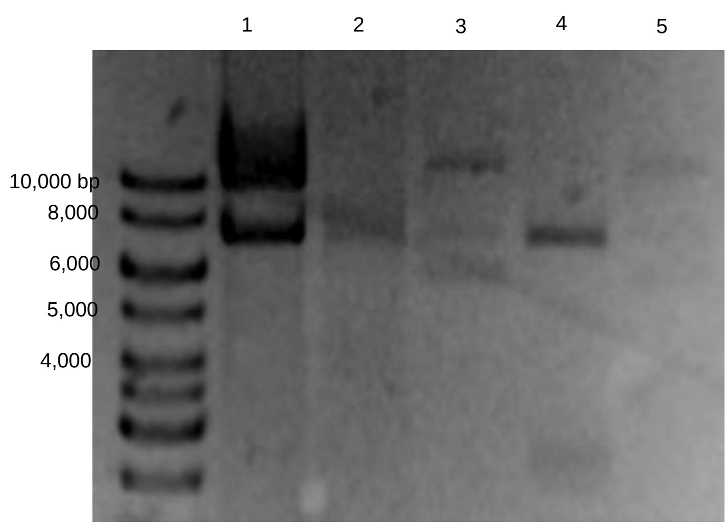

Figure 10. Double-digestion of isolated plasmids (from colonies A, B, and C) by restriction enzymes NdeI & XhoI. The double-digestion reaction was conducted as described in Experimental Procedures. Untreated circular vector pTYB2 (lane 1), double-digested vector pTYB2 (lane 2), double-digested plasmid from colony A (lane3), colony B (lane 4), colony C (lane 5). All 3 colonies were double-digested by NdeI and XhoI restriction enzymes for 3 hours.

All three of the purified plasmids from colony A, B, and C were double-digested by restriction endonucleases NdeI and XhoI at 37^0C for 3 hours and then were analyzed by 1% agarose gel electrophoresis (Figure 10) showing that colony B could be the pTYB2_*Uvr*A construct.

The possible construct of pTYB2_*Uvr*A (Colony B) was further amplified by transformation into *E. coli* DH5α competent cells. The plasmids from AMP^R transformants (colonies B1 and B2) were isolated, analyzed by double-digestion with restriction endonucleases Nde I and Xho I, and analyzed by 1% agarose gel electrophoresis (Figure 11). The plasmids from both transformants (colonies B1 and B2), the same as their original plasmid purified from colony B, were all very likely the construct of pTYB2_*Uvr*A.

The final construct of pTYB2_*Uvr*A was confirmed by DNA sequencing that revealed that both C and N-terminal junctions' sequences between inserted *Uvr*A fragment and the prepared vector pTYB2 were correct. The plasmid of pTYB2_*Uvr*A construct was transformed into C41 (DH3) *E. coli* competent cells for protein overexpression.

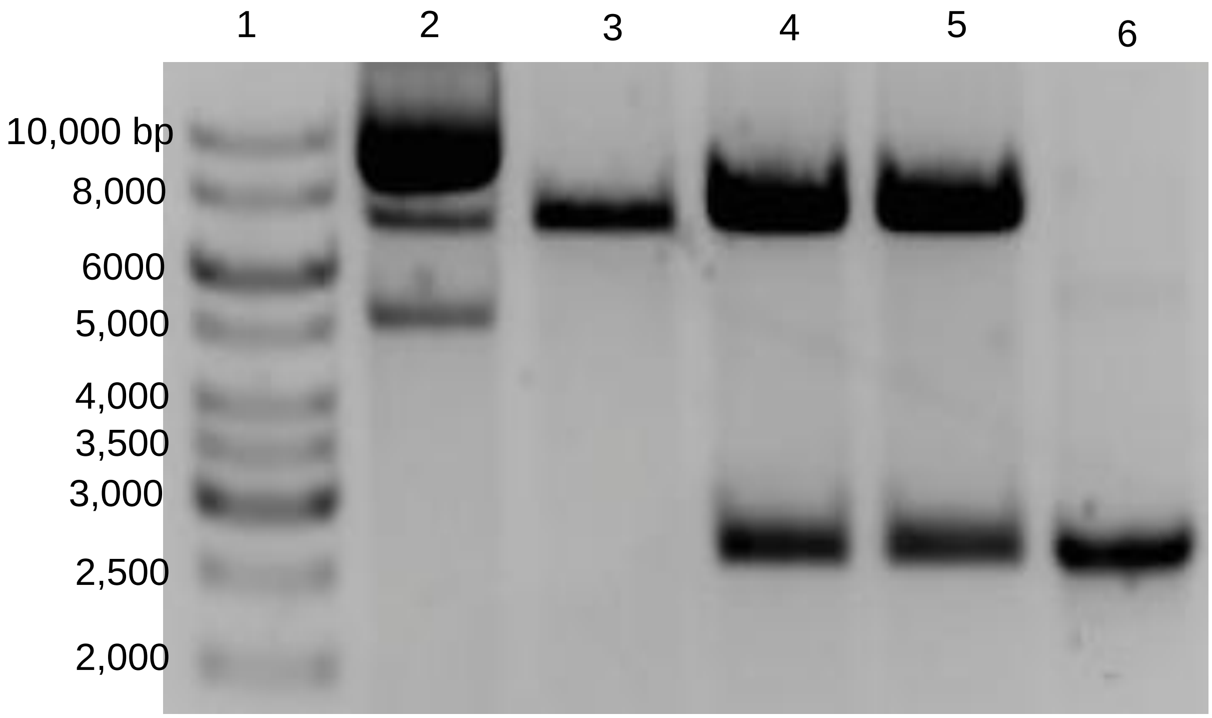

Figure 11: Confirmation of the amplified construct of pTYB2_*Uvr*A with restriction enzymes Nde I & Xho I. DNA ladder (Lane 1). Circular vector pTYB2 plasmid (Lane 2). Double-digested vector pTYB2 with NdeI & XhoI (Lane 3). Double-digested plasmid (from colony B1) with Nde I & Xho I (Lane 4). Double-digested plasmid (from colony B2) with Nde I & Xho I (Lane 5). Protein *Uvr*A from pool-32B (Lane 6).

Optimizing the Time Course of Induction of *Uvr*A Overexpression by IPTG

In order to determine the time course of induction, 120 ml culture in the flask was grown at 37^0C in LB medium until the OD_{600} reached 0.6 (15 ml aliquot were removed as the negative control). Then 0.7 mM IPTG was added to the medium and the culture was continued at 30^0C. The 15 ml samples were taken every hour from 3 hours through 7 hours. As indicated below in the Figure 12, the maximum volume of *Uvr*A overexpression was found to be attained 6 hours after the induction of 0.7 mM IPTG.

Visual examination of the gel by Coomassie blue staining (Figure 12) reveals that *Uvr*A protein production increased until a maximum level was reached at 6 hours post 0.7 mM IPTG induction. It inferred that a minimum culture time of 6 hours after induction will produce a substantial amount of the full-length *Uvr*A_intein fusion protein. Longer culture time may not result in any significant increase in yield.

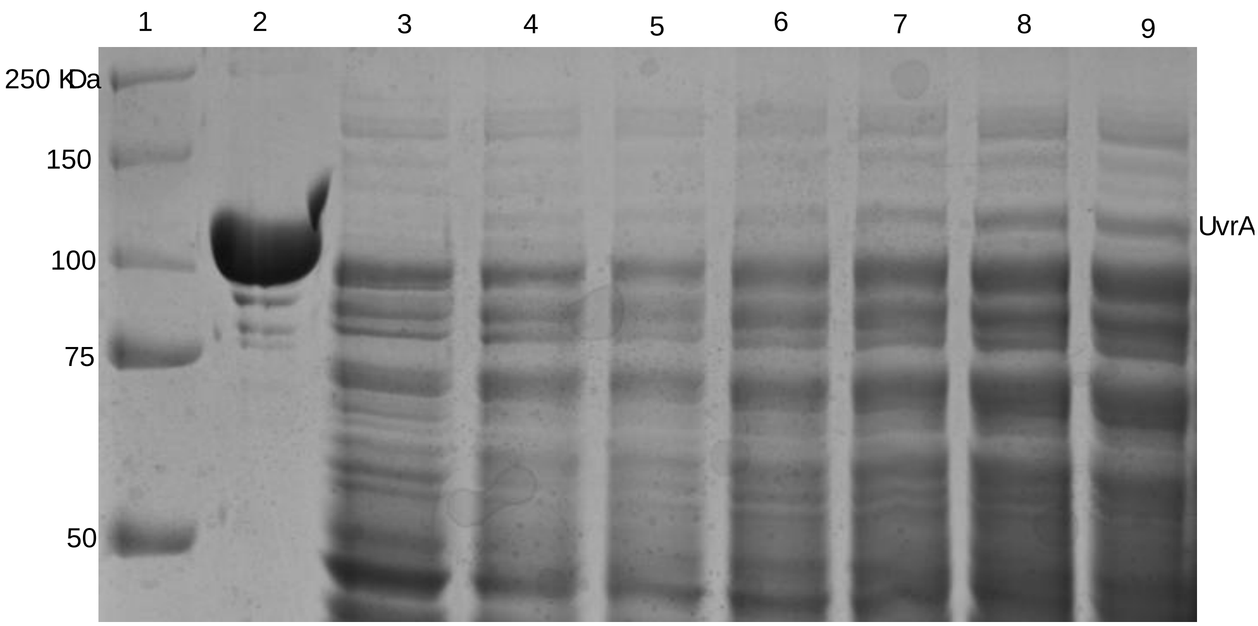

Figure 12: Optimization of the time course for the overexpression of *Uvr*A protein. Protein Marker (Lane 1). Protein *Uvr*A (from pool- 32B) (Lane 2). Clarified crude extract from uninduced cells (Lane 3). Clarified crude extract from cells, induced at 30°C for 2 hours (Lane 4), 3 hours (Lane 5), 4 hours (Lane 6), 5 hours (Lane 7), 6 hours (Lane 8), 7 hours (Lane 9). 15 ml aliquots were removed at indicated intervals.

Small Scale of *Uvr*A Purification by Chitin Beads to Check DTT Cleavage Efficiency

0.7 mM IPTG induction of *Uvr*A_Intein fusion protein in 60 ml culture and 30 ml culture without IPTG were performed at 30^0C for 6 hours with shaking (200 rpm). The resulting 60 ml induced culture then was split into 2 of 30 ml tubes, and the cells from 3 sample tubes were harvested separately by centrifugation at 6,000 rpm for 10 minutes at 4^0C. Pellets were resuspended in 1 ml lysis buffer for each sample with 1 mM PMSF and lysed by sonication (10 sec on, 10 sec off for 10 times). Clarified extracts (1 ml of each) were incubated with 50 µl chitin beads separately on a rotating rack for 2 hours. Recovered beads had 30 mM DTT added into one of the tubes that was from IPTG induction culture, and all 3 tubes of beads incubated at 4^{0}C overnight. All 3 samples from recovered beads and 3 supernatant tubes from the beads were analyzed by 10% polyacrylamide gel at 150 volts for 70 minutes and stained in Coomassie blue (Figure 13). Comparing the samples from the recovered beads from the extracts of culture with both 0.7 mM IPTG and 30 mM DTT (Lane 4) to that from the extracts of culture with 0.7 mM IPTG but without DTT (Lane 5), the cleavage of the protein *Uvr*A from its intein tag in the sample with 30 mM DTT overnight incubation was most efficient as Figure 13 indicated.

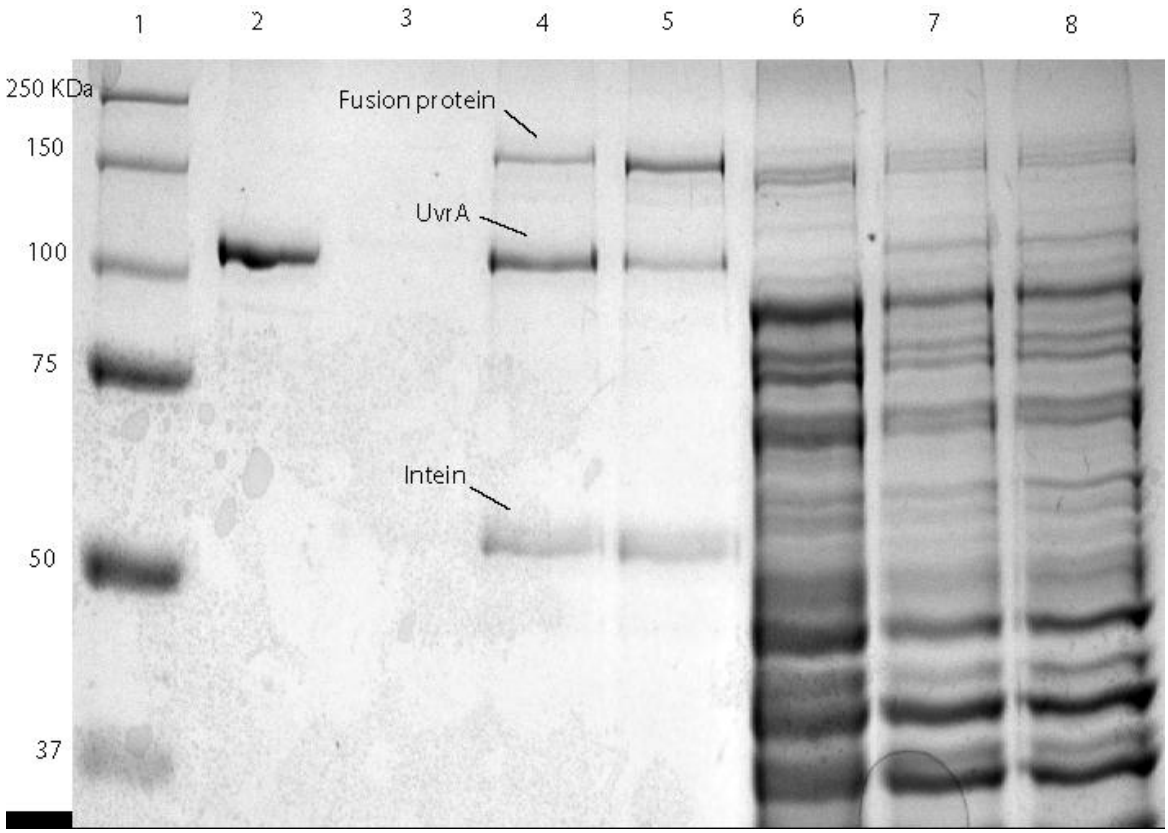

Figure 13: DTT cleavage efficiency for the binding between fusion protein and chitin beads. Protein Marker (Lane 1). Protein *Uvr*A (Lane 2). Recovered beads from the extracts of culture w/o IPTG induction (Lane 3). Recovered beads from the extracts of culture with 0.7 mM IPTG and 30mM DTT (Lane 4). Recovered beads from the extracts of culture with 0.7 mM IPTG but without DTT (Lane 5). Supernatant from sample of lane 3 (Lane 6). Supernatant from sample of lane 4 (Lane 7). Supernatant from sample of lane 5 (Lane 8). Small-scale *Uvr*A_intein overexpression under 0.7 mM IPTG induction. 1 ml clarified crude extracts (from 30 ml of non-induced/induced cells) incubated with 50 ul of chitin beads at 4°C for 4 hours. Then, all the beads were recovered by centrifugation at 2,500xg for 20 seconds. 30 mM DTT was only added into one of the recovered beads tubes incubated with the extracts from induced culture. All 3 recovered beads tubes were incubated at 4°C overnight.

Cell Lysis Efficiency Comparison Between Sonication and French Press

Different methods were used for the preparation of cell lysates from *E. coli* cells: sonication and French press. Sonication is the most popular technique for lysing small quantities of *E. coli* cells such as 1 L of cell culture. Cells are lysed by liquid shear and cavitation. French presses are the alternative devices to lyse bacteria *E. coli* cells. Cells are lysed by a liquid shear created by pressuring the cell suspension and suddenly releasing the pressure.

As indicated in Figure 14A, after the sonication (10 cycles of 10 sec pulses with 10 sec pauses), most of the UvrA protein is still in the pellets compared to that in the clarified extracts.

After using the French Press (Figure 14B) for the cell lysis, the most of the *Uvr*A protein shows in the clarified extracts instead of in the pellets.

The French press (model no. OMFA078A) was operated at 8,000 psi to achieve more adequate *E. coli* cells lysis compared to the sonication method which had much lower cell lysis efficiency in this case.

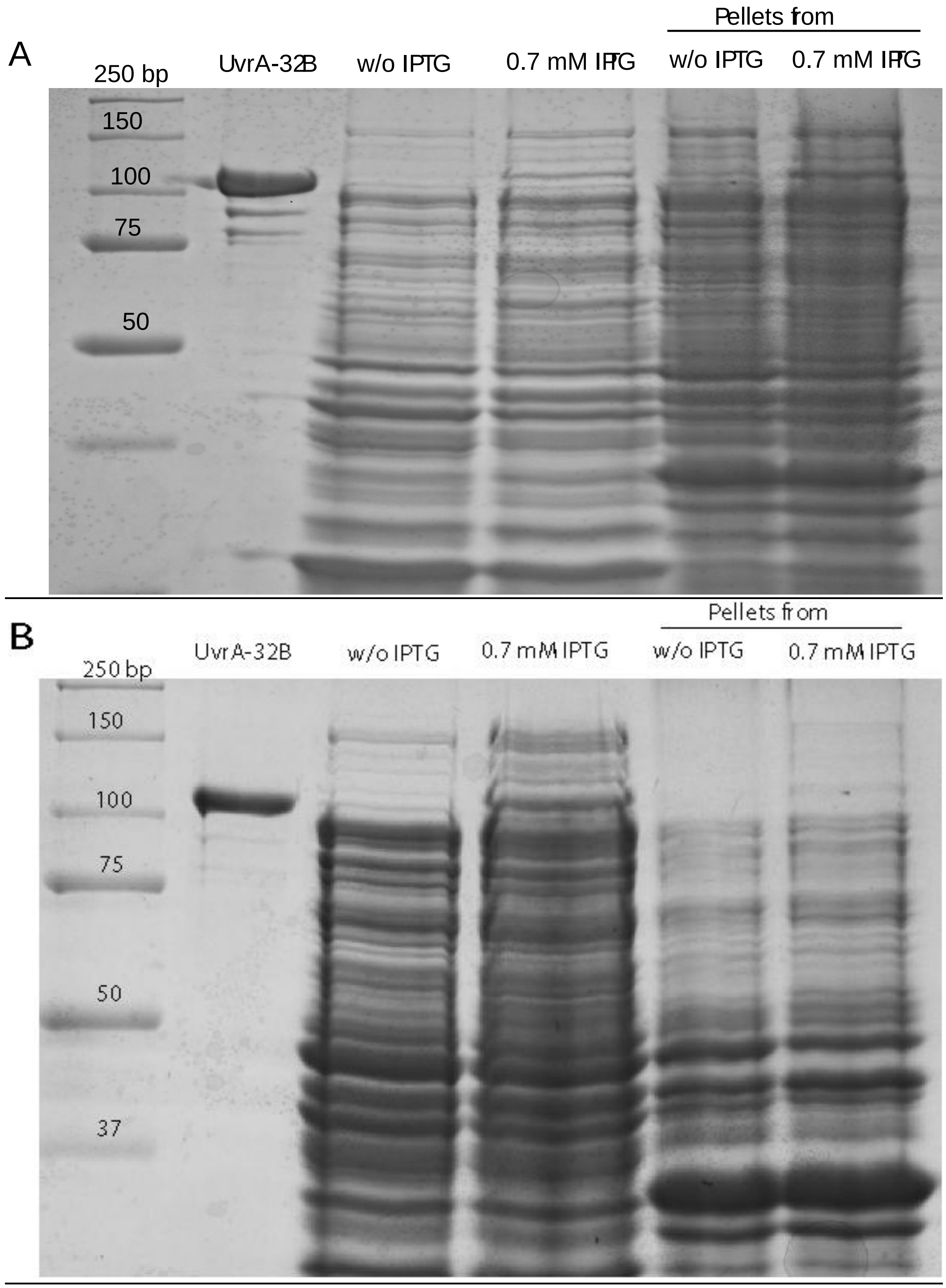

Figure 14: Comparison of cells lysis effects between the methods of sonication and French press. (A) After the sonication, most of the *Uvr*A protein is still in the pellets compared to the amount of the *Uvr*A protein in the extracts. (B) After using the French press for the cell lysis, most of the *Uvr*A protein shows in the extracts compared to the amount of *Uvr*A protein in the pellets.

Purification of *Uvr*A Protein from One Liter of Culture by T7 IMPACT System

For overexpression of *Uvr*A protein from C41_pTYB2_*Uvr*A *E. coli* cells, the fresh Amp^R transformants were inoculated into one liter of LB supplemented with ampicillin to a final concentration of 100 µg/ml. The culture was first incubated at 37^0C to an $O.D_{600}$ of 0.6, and then the IPTG was added into it to a final concentration of 0.7 mM followed by induction at 30^0C for 6 hours. The cells were harvested, and the pellets were resuspended in 80 ml column buffer with 1 mM PMSF and lysed by one pass through French press at 8,000 psi. The clarified extracts were slowly loaded onto the chitin columns (10 ml) at a rate no faster than 0.5 ml/min. After the column was washed by 30 column volumes of column buffer, on-column cleavage reaction was induced by flushing the column with 3 column volumes of cleavage buffer with 30 mM DTT. The flow in the column was stopped and left at 4 ^{0}C overnight. Protein *Uvr*A was eluted by cleavage buffer without DTT and was collected in 1 milliliter fractions of each for a total of 20 milliliters.

As indicated in Figure 15, fraction 2 and fraction 4 (fractions are 1 ml each for 20 ml in total after cleavage with DTT and elution from the chitin column) were examined using PAGE for molecular weight and purity of isolated protein. Other samples including some major steps from the overproduction and purification of *Uvr*A protein were also analyzed on a 10% SDS-polyacrylamide gel electrophoresis stained with Coomassie Blue and photographed. Such samples included those from cell extracts of noninduced cultures, 0.7 mM IPTG-induced cell cultures, the flow-through of extract loading, column washing, and on-column cleavage induction, and chitin resin, respectively. Fractions 1, 3,5,7,9 were also examined by 10% SDS-polyacrylamide gel electrophoresis stained with Coomassie blue for *Uvr*A molecular weight and purity of isolated protein *Uvr*A (Figure 16).

Fractions 1-10 and 11-20 were split into 2 dialysis tubings, pooled as pool-1 and pool-2 (as indicated in the Figure 17), and dialyzed against 1 liter of 50% glycerol storage buffer in the same beaker at 4^0C for 20 hours. The concentrations of protein *Uvr*A of both samples from pool-1 and pool-2 were measured by Pierce 660 nm Protein Assay, and they were 2.0 µM and 1.3 µM, respectively. The obtained purified UvrA protein from 1 liter of culture cells then was aliquoted into several of 1.5 ml microcentrifuge tubes and stored at -20^0C before its biological activity was confirmed.

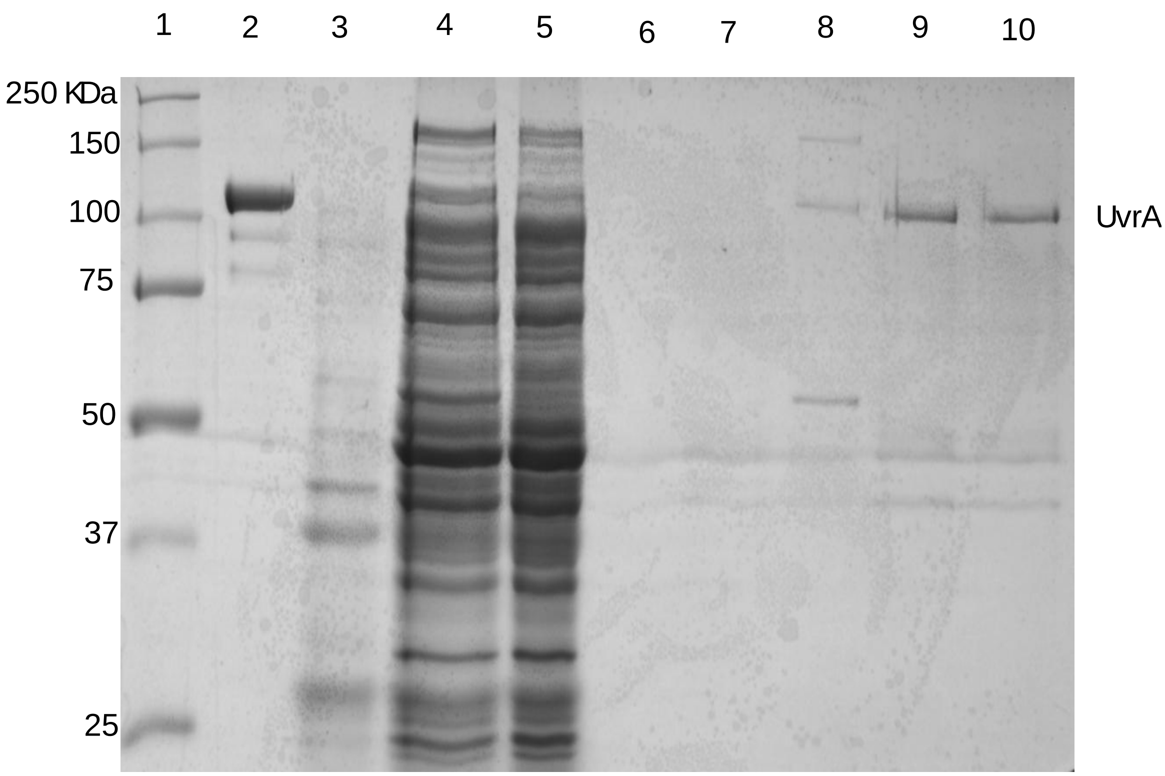

Figure 15: Expression and purification of the *Uvr*A protein using the IMPACT system. Protein marker (Lane 1). Protein UvrA from pool- 32B (Lane 2). Clarified crude extract from uninduced cells (Lane 3). Clarified crude extract from cells, induced at 30°C for 6 hours (Lane 4). Chitin column flow through (F.T.) (Lane 5). Chitin column wash (Lane 6). Quick DTT wash to distribute DTT evenly throughout the chitin column (Lane 7). The supernatant (Lane 8) from chitin resin, to determine the cleavage efficiency. Fraction 2 and 4 of eluted *Uvr*A after stopping column flow and inducing a self-cleavage reaction at 4°C overnight (Lane 9-10). Coomassie blue staining.

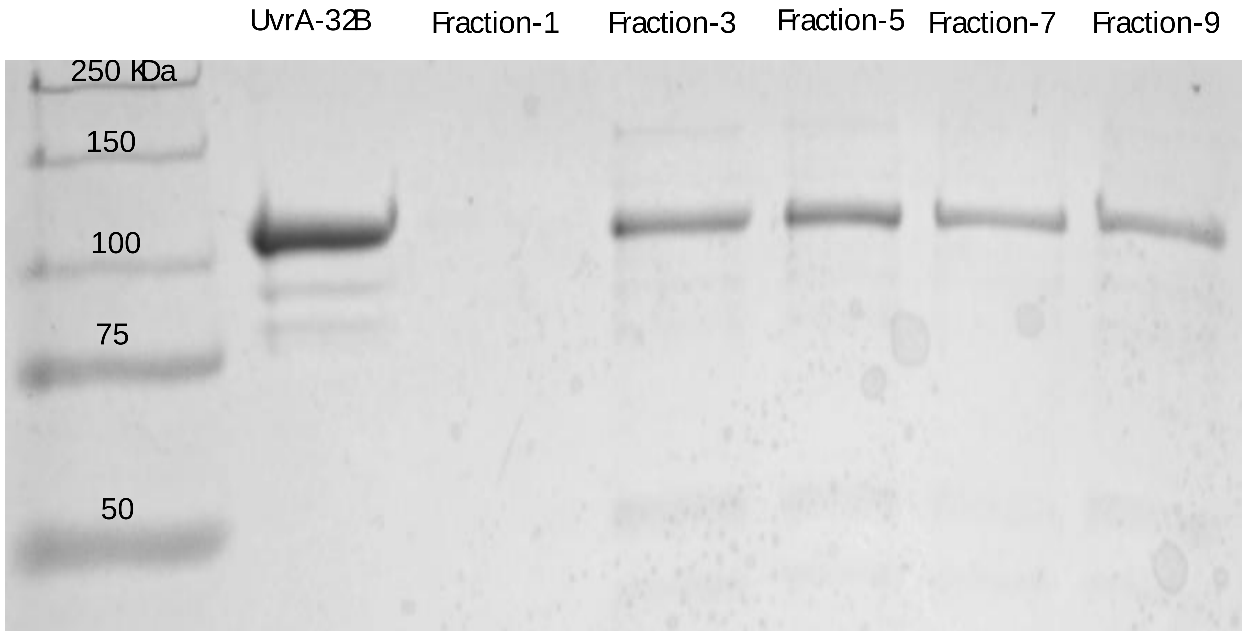

Figure 16: Comparison among the fractions of the eluted target protein *Uvr*A. 1 ml fractions of each for 20 ml in total collection of eluted target protein *Uvr*A.

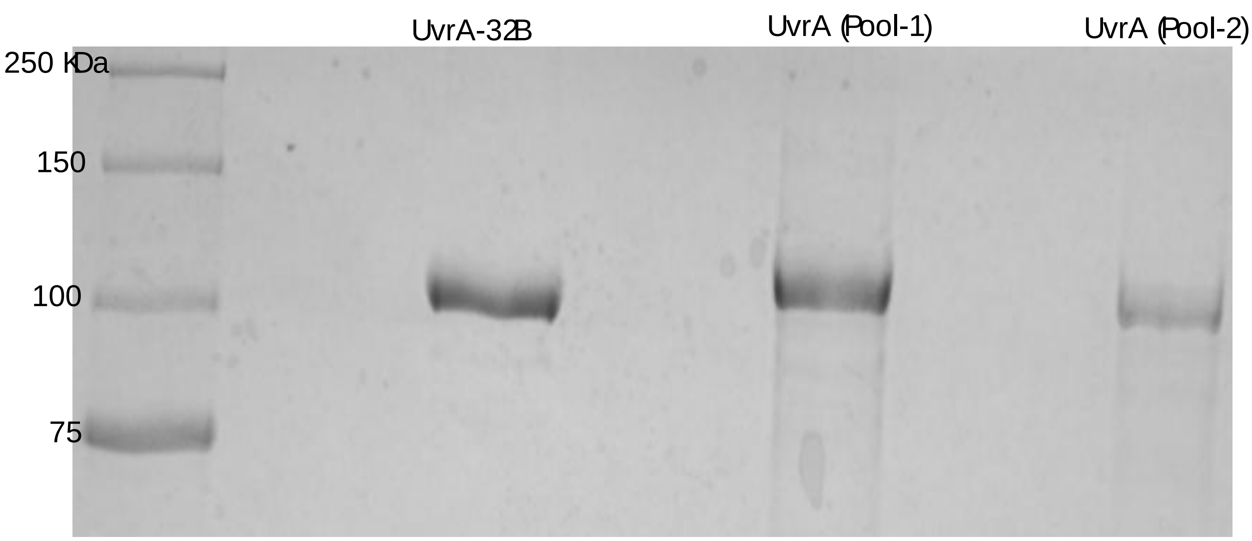

Figure 17: Purified protein *Uvr*A samples from pool-1 and pool-2. Purified *Uvr*A protein, from one liter of culture cells, split into two dialysis tubings (fractions 1-10 as pool-1 and 11-20 as pool-2), was dialysed against the 50% glycerol storage buffer for 20 hours at 4 ^{0}C. The concentrations of protein *Uvr*A from both samples of pool-1 and pool-2 were measured by Pierce 660 nm Protein Assay. The concentrations of the samples from pool-1 and pool-2 were 2.0 µM and 1.3 µM, respectively.

*Uvr*ABC Incision Assay to Characterize the Purified *Uvr*A Protein *in vitro*

^{32}P-labeled 58-bp duplex DNA substrate containing FABP [N-(20-deoxyguanosin-8-yl)-4-fluoro-4-aminobiphenyl] adducts was incubated with DNA excision nucleases *Uvr*A, B, and C. As indicated in Figure 18, the DNA incision products were present in the *Uvr*ABC DNA incision assay using the newly purified *Uvr*A protein. In agreement with these incision data, the result supported our hypothesis that prokaryotic DNA excision nuclease *Uvr*A protein can be purified by T7 IMPACT protein purification system.

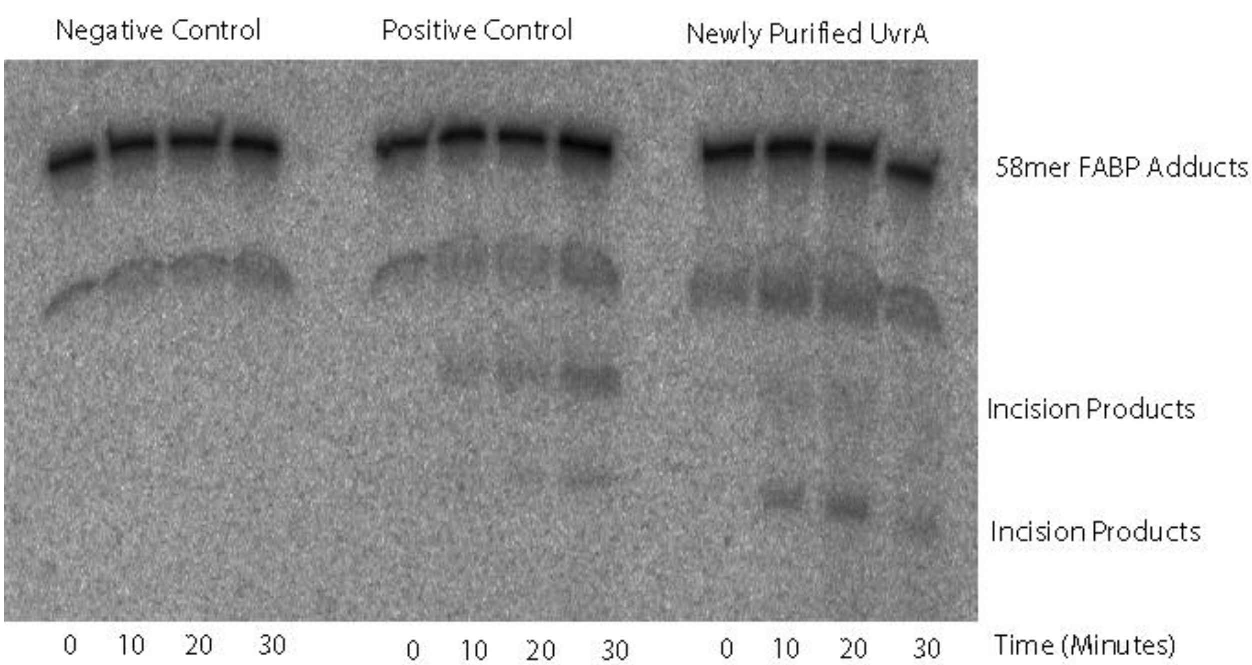

Figure 18: *Uvr*ABC incision assay. DNA substrates, ^{32}P-labeled at the 5' termini, were incised by *Uvr*ABC in *Uvr*ABC buffer at 37^0C for a specified length of time. Aliquots were collected at 0, 10, 20, and 30 minutes from the reaction. The reaction was terminated by heating at 95^0C for 5 minutes. The products were denatured into single strands by addition of formamide loading buffer and heating to 95^0C for 5 minutes, and immediately plunged into ice to prevent reannealing. Digested (incision) products were then analyzed by gel electrophoresis on a 12% polyacrylamide sequencing gel under denaturing conditions with TBE buffer. ^{32}P-labeled incision products or nonincision DNA bands in the gels were visualized using a Fuji FLA-5000 image scanner with MultiGauge V3.0 software.

CHAPTER 4

DISCUSSION

To express a target protein as a fusion protein to an affinity tag, such as GST (Glutathione *S*-transferase), MBP (Maltose-Binding Protein), or His (histidine)-tag systems, is a commonly used technology for recombinant protein expression and purification (LaVallie and McCoy 1995). Some affinity tags even can help to elevate the expression level of the fusion protein and thus the yield of the target protein (LaVallie and McCoy 1995). This technology usually requires a separate protease to cleave the target protein from its affinity tag (Chong et al. 1998). The proteases treatment creates an extra step to the protein purification, and proteases are sometimes nonspecific and inefficient, which could become a potential limitation of this technology (Chong et al. 1998). We need good quality of *Uvr*A, *Uvr*B, and *Uvr*C proteins for our research study in NER pathway. The prokaryotic DNA excision nuclease *Uvr*A we currently are using in our lab was purified previously by 4-column steps methods including Q-sepharose column, DNA cellulose column, Mono-Q FPLC column, and G-200 FPLC column. These experimental procedures are very time-consuming and technically challenging. In this project IMPACT was used as the cloning and protein purification system to purify a native recombinant UvrA in a single-column (single-step). The protein splicing element intein of IMPACT is able to cleave a peptide bond without the use of a separate protease, and the intein-catalyzed peptide bond cleavage eliminates the need for a protease normally required by other affinity fusion systems.

To subclone *Uvr*A DNA fragment, the C-terminus pTYB2 expression vector was chosen in the intein-tagged fusion protein construction with DH5α *E. coli* cells as the host organism. To prepare for the *Uvr*A DNA fragment to be cloned, PCR method was used for the amplification of *Uvr*A DNA fragments prior to the molecular cloning using pSST10_*Uvr*A as the template. Both the cloning vector pTYB2 and *Uvr*A DNA fragments were treated with 2 restriction endonucleases Nde I and Xho I, and these 2 restriction sites were used for the cloning of the amplified target gene of *Uvr*A in fusion construction. These 2 cleavage sites are unique within the multicloning site (MCS) of vector pTYB2 and noncutters within *Uvr*A fragment so that the vector can be cleaved only at a single site. To increase the recombination efficiency, the cleaved vector of pTYB2 was treated with an enzyme (alkaline phosphatase) that dephosphorylates the vector's ends. The vector molecules with dephosphorylated ends are usually unable to self-ligate. The DNA fragments prepared from the vector pTYB2 and *Uvr*A gene from pSST10_*Uvr*A plasmid were simply mixed together at 1:3 molar ratio and incubated with T4 DNA ligase to form 2 covalent phosphodiester bonds between 3' hydroxyl end of one nucleotide with the 5' phosphate end of another. The resulting DNA mixture that contained both correctly joined ends and randomly joined ends was sorted out by selectable marker and plasmid isolation after being transformed into the host organism DH5α *E. coli* cells. The vector pTYB2 has a gene that confers resistance to ampicillin. Cells harboring the pTYB2 will survive and grow when exposed to the ampicillin, while those that have failed to take up pTYB2 sequence will die. The desired products with vector pTYB2 covalently linked to *Uvr*A DNA would be present, but undesired side products could be present also. To identify the organisms containing desired *Uvr*A DNA inserts (recombinant DNA of pTYB2 and *Uvr*A), it is necessary to examine a number of different clones (by restriction fragment analysis, polymerase chain reaction, and/or DNA sequencing) to be sure that the desired pTYB2_*Uvr*A DNA construct was obtained. One clone was selected and sequenced to confirm that the sequences at both junctions of the *Uvr*A fragment insertion into the pTYB2 vector were correct.

Overexpression of the fusion protein from pTYB2 vector may be affected by the following factors: (A) *E. coli* strain; (B) cell culture conditions (cell density, temperature, etc.); (C) protein expression induction conditions (the IPTG concentration, temperature, duration, etc.). To achieve a maximal overexpression of fusion protein of *Uvr*A_intein, C41 (DE3) *E. coli* strain derived from BL21 (DE3) (Miroux and Walker, 1996) was used. There is at least one uncharacterized mutation in the C41 strain. Compared to the BL21 (DE3) strain, C41 (DE3) cells are believed to increase the activity and/or amount of T7 RNA polymerase to have a higher expression level and prevent cell death associated with expression of many recombinant toxic proteins. The *Uvr*A protein yield seems intermediate, and I may need to further optimize some other protein expression induction conditions, such as temperature, IPTG concentration, and buffer's pH levels, etc.

The pTYB2_*Uvr*A plasmid was transformed into C41 *E. coli* cells, and a small scale induced culture at 30^0C with 0.7 mM IPTG showed a maximum level of the fusion protein *Uvr*A_intein after 6 hours. In our lab purifications of the prokaryotic DNA excision nuclease *Uvr*B and *Uvr*C were successfully performed previously using pTYB2 vector. Most of the cell culture and protein expression conditions are similar among these 3 kinds of proteins, except the IPTG induction time course for *Uvr*A, *Uvr*B, and *Uvr*C overexpression are different, and they are 6 hours, 2.5 hours, and 3 hours, respectively. The cell extract from 1 liter culture of C41(DH3)_pTYB2_*Uvr*A *E. coli* cells induced under 0.7 mM IPTG at 30^0C for 6 hours was applied onto the chitin column, and the cleavage of the induced fusion protein of *Uvr*A_intein was induced using cleavage buffer containing 30 mM DTT at 4^0C overnight. The eluted *Uvr*A protein was dialyzed against the storage buffer with 50% glycerol at 4^0C and stored at -20^0C for a short time period and -80^0C for long-term storage. The *Uvr*ABC incision assay results showed that the *Uvr*A protein purified by T7 IMPACT system is biologically active, which demonstrated that IMPACT system can be used for the purification of active *Uvr*A protein in a single step. Our hypothesis for this project that prokaryotic DNA excision nuclease *Uvr*A protein can be purified by T7 IMPACT system was supported by our experimental data.

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