ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 7, Issue 1, pp: (50-53), Month: January - March 2019, Available at: www.researchpublish.com

Cloning, Expression, and Purification of Histidine-Tagged *Wolbachia* surface protein

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Abstract: ***Wolbachia*** **surface protein (WSP) is an Outer membrane protein of gram negative bacteria** ***Wolbachia*****. WSP has shown its functional role in immune response, cell proliferation, pathogenicity and controlled cell death program. The WSP gene has previously been cloned, but the expression of the protein is low and the purification is time consuming. Therefore the** ***E. coli*** **WSP gene was cloned into the pET19b vector to improve the protein expression and simplify the purification. The WSP gene sequence was utilized to design forward and reverse oligonucleotide primers that were used to PCR the gene from** ***Wolbachia*** **genomic DNA. The primers were designed with NdeI or Xho 1 restriction sites on the 5'and 3' terminus respectively. The PCR product was sequenced to confirm the identity of WSP. The gene was cloned into the expression vector pET19b through NdeI and Xho 1 restriction endonuclease sites. The resulting plasmid containing WSP was transformed into the bacterial strain BL21 (DE3).The transformed cells were utilized to grow and express the histidine-tagged and the protein was purified using Ni-NTA affinity chromatography.SDS/PAGE gel analysis has shown that the protein was 69% pure and has approximate subunit molecular weight of 25 kDa. The protein purification is completed in one day and 1 liters of culture produced approximately 18 mgs of protein, an improvement on the previous protein expression and purification.**

Keywords: ***Wolbachia*** **surface protein (WSP), Apoptosis, Protein expression and purification,** ***E.coli*** **strain BL21 (DE3), Ni-NTA affinity chromatography.**

1. INTRODUCTION

Wolbachia surface protein (WSP) was isolated from *Wolbachia* bacteria in 1998 by Braig HR (Braig, H. R et al. 1998).The *Wolbachia* has seven outer membrane proteins (OMPs) and WSPs in *B. malayi* and highly conserved in *Wolbachia* from filarial nematodes and have a heterogeneous pattern of amino acid diversity characteristic of other OMPs known to be involved in bacteria-host interactions in other systems (Baldo L et al. 2005, Baldo L et al. 2010, Braig, H. R et al. 1998) since then the protein from several different species like B.malayi has been isolated and characterized (E.Melnikow et al.2013). *Wolbachia* WSP gene encodes a polypeptide of 245 amino acid residues. The resulting protein has a molecular weight of 25K Daltons.WSP is one of the most translated membrane protein of *Wolbachia* present in *Drosophila* host with single gene copy. The bacterial wsp protein undergoes frequent recombination, and the scientist all over the world suspect the protein for being responsible for the reproductive anomalies. Extensively, in vitro studies demonstrate that WSP inhibits apoptosis of PMN cells (Bazzocchi C. et al. 2007). WSP has also been shown to function as a ligand of Toll-Like Receptors (TLR2), a receptor that controls apoptosis of PMNs and other types of host cells (Francois et al. 2005). Studies have also shown that WSP is a strong immune elicitor (Sofia B Pinto et al. 2012)

Previously, the WSP gene from nematode hosts was cloned into the plasmid vector pET19b and transformed into an *E. coli* strain in order to characterize and apoptosis human neutrophils (Bazzocchi C. et al. 2007). However, cloning of the WSP gene from E. coli into a histidine-tagged (His-tag) expression vector from Drosophila host had not been done before. In this paper we report the cloning of the *E. coli* WSP gene into the pET-19b plasmid vector next to the six His-tag sequence and transformed into the *E. coli strain BL21* (DE3) that is ideal for protein expression (Kurz et al. 2008).

2. MATERIALS AND METHODS

Enzymes for DNA cloning and amplification were purchased from 5 Prime Eppendorf (Hamburg, Germany). The plasmid pET19b was from Novagen (Madison, WI). Bacterial strain BL21 (DE3) was from Invitrogen and manipulated and maintained using standard techniques. The Ni-NTA affinity matrix was purchased from Qiagen (Valencia, CA). Buffers and all other chemicals were purchased from Hi Media (India). Oligonucleotides for the cloning of the WSP gene were synthesized by Chromos Laboratory Bangalore India. IPTG was from Bangalore Genei Pvt Ltd (India).

Cloning of *WSP* into the pET-19b vector

Proximity of the *WSP* gene to the His-tag sequence of pET-19b was kept by utilizing the restriction enzymes NdeI and XhoI. The forward (5'- C**GAATTC**ATATGGATCCTGTTGGTCCAATAAGTG-3') and reverse (5'- G**CCTCGAG*TCT AGA***CCTAGAAATTAAACGCTAC -3') 30-mer primers for the *WSP* gene were designed accordingly. Chromosomal DNA was obtained from an overnight culture of *E. coli* strain JM109 and isolating the genomic DNA using Phenol-Chloroform: IAA purification method (Chomczynski and Nicoletta Sacchi, 1987). The *WSP* gene was obtained by PCR using 1 µg of chromosomal DNA. Thirty five cycles of PCR were done using 1 Unit of Taq DNA polymerase (5 Prime Eppendorf). The PCR product was cleaned with the Chromous PCR Cleanup kit and subsequently digested by NdeI and XhoI restriction endonuleases. The pET-19b vector was also digested with NdeI/ XhoI restriction endonucleases and cleaned using the same kit. The digested plasmid and PCR product were ligated with T4 DNA ligase resulting in the pWSP plasmid. The plasmid was transformed into the bacterial strain BL21 (DE3) using standard procedures. The transformed cells were plated on LB plates containing ampicillin (50 µg/ml) and incubated overnight at 37°C. Each plate contained about 50 colonies. The culture plates were stored at 4°C.

Ten colonies were picked from one of the plates and used to inoculate 5 mL of autoclaved LB broth containing 50 µg/ml ampicillin. The cultures were grown overnight at 37°C with shaking at 250 rpm. The pWSP plasmids were isolated using a Chromos Mini-Prep kit. Each plasmid sample was subjected to digestion by NdeI and a double digestion by both NdeI and XhoI. Agarose gel electrophoresis was carried out on the digested products to confirm an approximately 650 bp insert, the expected size of the WSP gene. Six out of 10 colonies contained the insert.

3. PROTEIN EXPRESSION AND PURIFICATION

The positive clones were transformed into an expression host Escherichia coli BL21 (DE3) pLys (Invitrogen) and induced with 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated for 2hrs at 37˚C. Cells were harvested and 100 ml lysis buffer (10mMTris-Cl pH-7.5, 50mM NaH2PO4, 200mM NaCl, 8M Urea, 10mM Imidazole) was added per 2L pellet. To induce lysis the cells were re-suspended and the pellet was sonicated for 30-45 min at constant duty cycle and output energy of 80W. The cell lysate was spun down for 10,000 RPM for 15 mins and incubated for 5 min with 2ml of Ni-NTA column matrix. The protein bound was transferred to a column and washed extensively with wash buffer (50mM NaH2PO4 pH- 7.5, 200mM NaCl, 8M Urea, 20mM Imidazole) and collected washes as Wash I (2ml) and Wash II (18ml). Bound protein was eluted using 7.5ml of gradient elution buffer (20mM Tris-Cl pH- 7.5, 200mM NaCl, 8M Urea 30mM-300mM Imidazole). 31 fractions of 0.5ml each was loaded on to 12% SDS-PAGE and electrophoresis was done at 100V for 4 hrs and stained (Kurz et al. 2008).

4. RESULTS AND DISCUSSION

The expression of WSP was attempted by directing it to the cytoplasm through the removal of the signal sequence. The cytoplasm- directed WSP was expressed in insoluble form. The protein was detected by SDS Page in the cytoplasmic pellet and was confirmed that *Wolbachia pipientis* surface protein without the signal peptide is transported to the *E.coli* cytoplasm and then the expression was carried out in 2L culture. The positive clones were transformed into expression host (BL21DE3), and induced with 1mM IPTG final concentration and incubated for 2hrs at 37˚C. Cells were harvested and lysed with sonicator and loaded on 12% SDS-PAGE and electrophoresis at 100V for 4 hrs and stained with colloidal stainer. The WSP was expressed showing a band at ~25KD which is in excellent agreement with the theoretical and Insilco analysis (Uday et al 2012) in induced (lane 3-8 Figure.1) when compared to uninduced clones of Bl23 DE3 and DH5 alpha. Since the WSP was found to be insoluble, the purification was performed in different conditions i.e. Native (no UREA), denaturing (UREA) and denaturing with elution using pH variation. The purification was performed by decreasing the concentration of Urea from 4M to 0M. The denaturing purification method did work and purification was

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performed by denaturing condition. The initial purification step of WSP running immobilized metal affinity chromatography IMAC extracted 19 mg of 69%, pure protein from 1 L of culture (Table 1). High molecular mass contaminants were removed by size exclusion chromatography. This initial purification step yielded 18mg of homogeneous WSP from 1 L of culture (Figure. 1).

Table 1: Summary of the purification of recombinant WSP: Total protein was quantified using BCA protein assay with BSA as a standard.

Purification step	Total protein mg/ml	Total volume	Amount protein (mg/ml)	Yield in mg	Yield in %	Purity
Lysate	9.3	100	1.1	107	100	12
IMAC	3.5	8	2.4	19	18	69
Gel filtration	3.8	4.8	3.8	18	18	100

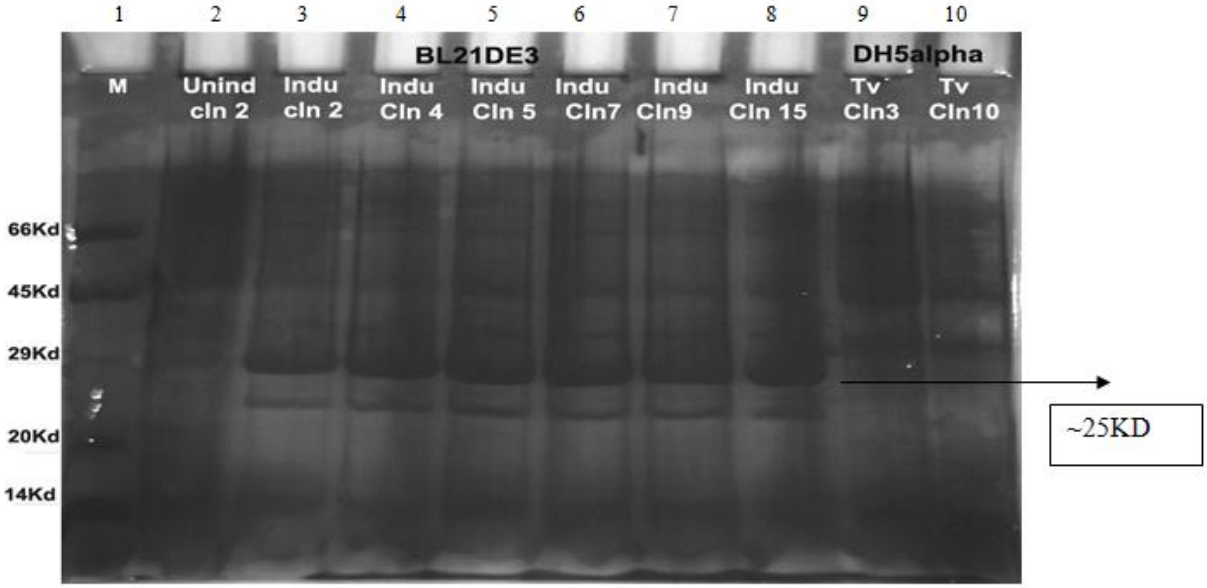

Figure 1: SDS PAGE Analysis of *Wolbachia* surface protein

The study presents the first report on amplification and cloning of the full-length WSP gene into pET19b; the protein was over expressed in *E. coli BL21DE3* cells. The expression was attempted by directing it in the cytoplasm through removal of the signal sequence. Although, WSP can be purified from periplasm, it is possible that there may be structural change in the helical domain of WSP, leading to the formation of inclusion bodies. The accumulation of inclusion protein is a common problem for heterologous protein expression and is often caused by incomplete translocation across the inner membrane (Baneyx, 1999). Therefore, it was attempted to extract the insoluble protein under denaturing conditions with urea or other reagents followed by refolding. Additionally, different incubation temperatures and IPTG concentrations were used to lower the rate of protein production, allowing the protein to fold correctly and avoid inclusion bodies. And thus WSP was successfully expressed and purified in soluble form.

ACKNOWLEDGMENTS

This work was supported in part by funds from Department of Atomic energy, Board of Nuclear Science and (Grant Sanction No.2012/37B/12/BRNS/953 dated 20/06/2012) Department of Life Science, Bangalore University, Bangalore.

Conflict of Interest

There is no Conflict of Interest for the Authors

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