New BIOTECHNOLOGY 46 (2018) 7–13

Contents lists available at ScienceDirect

New BIOTECHNOLOGY

journal homepage: www.elsevier.com/locate/nbt

Full Length Article

# Enhanced production and immunological characterization of recombinant West Nile virus envelope domain III protein

Nagesh K. Tripathi[a,*], Divyanshi Karothia[b], Ambuj Shrivastava[b], Swati Banger[a], Jyoti S. Kumar[b]

[a] Bioprocess Scale Up Facility, Defence Research and Development Establishment, Jhansi Road, Gwalior, 474002, India
[b] Division of Virology, Defence Research and Development Establishment, Jhansi Road, Gwalior, 474002, India

ARTICLE INFO

*Keywords:*
*Escherichia coli*
Recombinant protein
Bioreactor
Purification
Immunogenicity

ABSTRACT

West Nile virus (WNV) is an emerging mosquito-borne virus which is responsible for severe and fatal encephalitis in humans and for which there is no licensed vaccine or therapeutic available to prevent infection. The envelope domain III protein (EDIII) of WNV was over-expressed in *Escherichia coli* and purified using a two-step chromatography process which included immobilized metal affinity chromatography and ion exchange chromatography. *E. coli* cells were grown in a bioreactor to high density using batch and fed-batch cultivation. Wet biomass obtained after batch and fed-batch cultivation processes was 11.2 g and 84 g/L of culture respectively. Protein yield after affinity purification was 5.76 mg and 5.81 mg/g wet cell weight after batch and fed-batch processes respectively. The purified WNV EDIII elicited specific antibodies in rabbits, confirming its immunogenicity. Moreover, the antibodies were able to neutralize WNV *in vitro*. These results established that the refolded and purified WNV EDIII could be a potential vaccine candidate.

## Introduction

West Nile Virus (WNV), family *Flaviviridae*, genus *Flavivirus,* has become an important pathogen for public health and domestic animals [1]. The natural transmission cycle of WNV involves mosquitoes and birds, with humans and other mammals as incidental hosts [2]. To date, neither an approved human vaccine nor a suitable therapeutic are available against the infection [3,4]. The WNV genome comprises single-stranded positive-sense RNA of about 10.7 kb. The single open reading frame (ORF) encodes a polyprotein, which is enzymatically cleaved into three structural and seven nonstructural proteins. The structural proteins are the capsid (C), membrane (M) and envelope (E) proteins, and the nonstructural proteins are designated NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 [5]. The E protein is responsible for viral attachment, fusion, penetration, virulence and attenuation [6] and has three domains, I, II and III [7]. E protein domain III (EDIII) consists of about 100 amino acid (aa) residues, stabilized by a single disulfide bond. EDIII is the main immunogen generating neutralizing antibodies and provides protection against WNV [8–12].

In order to facilitate the development of a subunit vaccine, it is necessary to develop a large scale process to produce the protein in bulk with high immunogenicity. For vaccine development, specific antibodies against recombinant WNV EDIII must neutralize WNV. The recombinant E, EDIII and NS1 proteins have been produced using different hosts, including *Escherichia coli*, yeast, insect cells and plants [11–16]. *E. coli* is the preferred expression system for large scale production of various therapeutic, prophylactic and diagnostic proteins [17,18], but lacks post-translational modifications (PTM). Since EDIII is non-glycosylated, it can be successfully expressed in *E. coli.* Recombinant protein expression in *E. coli* is often associated with formation of inclusion bodies (IB) [19], which must be solubilized using high concentrations of chaotropes such as urea (8 M) or guanidine hydrochloride (6 M) to recover proteins for further purification [20].

Renaturation or refolding of recombinant proteins is usually carried out by removing denaturing or chaotropic agents *via* rapid dilution or pulse dilution, dialysis and diafiltration in the presence of additives such as arginine or redox forms of glutathione. However, refolding causes loss of protein leading to a low protein yield [17,20,21]. Liquid chromatography [affinity, ion exchange, hydrophobic interaction or size exclusion (gel filtration)] is an alternative technique for protein

*Abbreviations:* WNV, West Nile virus; EDIII, envelope protein domain III; C, capsid; E, envelope; NS, nonstructural; IB, inclusion bodies; IMAC, immobilized metal affinity chromatography; IEX, ion exchange; LB, Luria Bertani; TB, terrific broth; IPTG, isopropyl β-D-1-thiogalactopyranoside; WCW, wet cell weight; DCW, dry cell weight; DO, dissolved oxygen; VVM, gas volume flow per unit of liquid volume per minute; RPM, revolutions per minute; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; BCA, bicinchoninic acid; HRP, horseradish peroxidase; ELISA, enzyme linked immunosorbent assay; BSA, bovine serum albumin; PVDF, polyvinylidene fluoride; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; PBS, phosphate buffered saline; PRNT, plaque reduction neutralization test; PFU, plaque forming units; MEM, minimal eagle's medium; FBS, fetal bovine serum

* Corresponding author.
*E-mail addresses:* tripathink@gmail.com, tripathink@drde.drdo.in (N.K. Tripathi).

https://doi.org/10.1016/j.nbt.2018.05.002
Received 22 June 2017; Received in revised form 4 May 2018; Accepted 6 May 2018

refolding and is commonly used in place of dialysis or dilution type refolding. Immobilized metal affinity chromatography (IMAC) has become a technique of choice with the ability to perform simultaneous protein renaturation and purification leading to a high yield of product [20]. After primary purification using IMAC, further enhancement of protein purity could be achieved by ion exchange (IEX) or gel filtration chromatography. Previously, we reported the small scale expression of WNV EDIII in *E. coli*, with assessment of its immunogenic potential and protective efficacy in mice [22]. Here, we report a large-scale process for its production also in *E. coli*. Further, on-column refolding using IMAC and subsequent purification by IEX chromatography were carried out to achieve highly pure and immunogenic WNV EDIII protein. We also present data showing that the antibodies raised in rabbits against purified and renatured WNV EDIII neutralize the wild type virus. WNV EDIII, produced on a large scale and after two step purification, was found to be immunogenic

## Materials and methods

### *Microorganism, media and solutions*

*E. coli* strain BL21(DE3) was used as host for the expression of WNV EDIII. The EDIII gene of WNV (Eg101 prototype strain GenBank accession number AF2609681) was cloned into the *E. coli* expression vector pET 28a to yield the recombinant plasmid pET WNV EDIII as described earlier [22]. The transformed colonies *E. coli* were selected on kanamycin plates. Stocks of recombinant *E. coli* cultures were made in 50% glycerol (v/v) and stored at −80 °C. Luria Bertani broth and Terrific broth (BD Difco, USA) were used in shake flask cultures and batch cultivations. For fed-batch process, the initial batch medium contained Terrific broth (TB) and the feeding medium comprised yeast extract, 300 g and glycerol, 300 mL/L. Kanamycin (50 µg/mL) was used for all the shake flasks as well as bioreactor cultivations.

### *Shake flask culture for expression of recombinant WNV EDIII*

Small-scale shake flask culture was used for expression of WNV EDIII. For media optimization, *E. coli* cultures were grown in duplicate in 1L Erlenmeyer flasks containing 200 mL LB and TB medium at 37 °C with shaking speed of 180–200 rpm in an incubator-shaker (Kuhner AG, Switzerland). The primary inoculum was obtained on inoculation of 1.5 mL of *E. coli* stock culture in test tubes containing 10 mL LB medium and incubated at 37 °C and 150 rpm for 8 h. The primary inoculum [2% (v/v)] was then subcultured in 250 mL Erlenmeyer flasks with 50 mL of respective culture medium and kept at 37 °C overnight with shaking speed of 180 rpm. This overnight culture (4 mL) was used as the seed culture for media optimization. During the mid-log phase (~4–5 h of post inoculation), IPTG (1 mM) was added into the cultures to induce protein expression. The induced cultures were pelleted 4 h post-induction at 6000 rpm for 20 min at 4 °C and stored at −20 °C. The optical density ($OD_{600}$), wet cell weight (WCW), dry cell weight (DCW) and recombinant protein expression level were analyzed by sampling the aliquots of culture at different time intervals.

### *Batch bioprocess for production of recombinant WNV EDIII*

A laboratory scale bioreactor of 5 L working volume (Eppendorf, New Brunswick, USA) was used for bulk production of WNV EDIII using a batch cultivation process. The bioreactor vessel containing 5L TB medium was inoculated with an overnight grown culture (5% v/v) as described earlier. The temperature of the growth medium was maintained at 37 °C. Aeration and agitation rate were 0.5 to 1.0 vvm and 200 to 550 rpm respectively during batch cultivations. The dissolved oxygen (DO) concentration was maintained between 20 and 30% of air saturation using air, varying agitation rate (200 and 550 rpm) and supplying pure oxygen with air wherever necessary. The pH value was maintained at 7.0 using aqueous ammonia solution (25%) or phosphoric acid. Antifoam was added to prevent foaming. The batch cultures were grown until mid-log phase (5.5–6 h post inoculation) before induction and then inducer (1 mM IPTG) was added for expression of WNV EDIII. The cells were harvested 4 h post-induction using batch centrifugation for 30 min at 6000 rpm and 4 °C. During cultivations, sampling was performed to check culture growth by measuring cell density ($OD_{600}$).

### *Fed-batch bioprocess for production of recombinant WNV EDIII*

For fed-batch cultivations, the culture conditions were controlled similarly to the batch process. About 200 mL of overnight grown inoculum as described above was added into 4L TB medium in a bioreactor vessel. Temperature and pH were maintained as described above. Agitation rate was varied between 100 and 800 rpm and aeration was kept between 0.5 to 1.5 vvm to maintain DO between 20 and 30%. Pure oxygen along with air was also used at high cell density as required. At mid-log phase, when nutrients were depleted from the cultivation media as observed by an increase in DO value, feeding was initiated (~5 h post-inoculation). Feeding medium was pumped into culture medium in a linear manner (1 mL to 2 mL per min) over a 9.5 h period. When the DO level reached below the set value, feeding was terminated until the set value was achieved. Simultaneously, when the pH value dropped below the set value, feeding was stopped until the set value was obtained. During this fed-batch process, pH and DO stat feeding strategies were utilized. After 11 h cultivation, the culture was induced with 1 mM IPTG to express WNV EDIII. The cultivation process was continued until 4 h post induction before cell harvesting. Analysis of culture growth was carried out hourly. Cells were harvested and pelleted by centrifugation at 6000 rpm for 20 min.

### *Cell disruption and IB solubilization*

The cell disruption and IB solubilization were carried out as described earlier with minor modifications [23]. The cell pellet was washed twice with cell wash buffer (10 mM Tris–HCl, 100 mM NaCl, 10 mM EDTA, pH 8.0) (1:20, w/v) by centrifugation at 7000 rpm, 4 °C for 20 min. The washed cell paste was dissolved in chilled cell lysis buffer [10 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and lysozyme (100 µg/mL), pH 7.5)] (1:20, w/v). The cells were lysed by sonication (Sonics, USA) at 70% frequency. The sonicator was set to provide 9 s on and 9 s off pulses for 10 min. The lysate was further pelleted by centrifugation at 10,000 rpm, 4 °C for 40 min to obtain inclusion bodies (IBs). The IBs were washed by centrifugation with IB wash buffer 1 [50 mM $NaH_2PO_4$, 200 mM NaCl, 5 mM EDTA, 0.5 M urea and TritonX-100 (1%, v/v), pH 6.0] and further resuspended in IB wash buffer 2 (50 mM $NaH_2PO_4$, 1 M NaCl and 1 mM EDTA, pH 6.0) followed by centrifugation. The supernatant was discarded and the IBs were used for further purification. Solubilization of IBs was carried out using IB solubilization buffer (10 mM Tris–HCl, 100 mM NaCl, 100 mM $NaH_2PO_4$ and 8 M urea, pH 8.0) (1:20, v/w) by stirring on a magnetic stirrer at room temperature for 1 h. The solubilized IBs were then subjected to centrifugation. The pellet was discarded and clear supernatant was further used to purify WNV EDIII.

### *On-column refolding and immobilized metal affinity chromatography*

Affinity chromatography was performed employing an AKTA Explorer FPLC system or peristaltic pump (GE Healthcare, Sweden) for on-column refolding and primary purification of WNV EDIII. For this purpose, three buffers, namely A [300 mM NaCl, 50 mM phosphate, 3 M urea and glycerol (12%, v/v), pH 8.5], B (buffer A + 50 mM imidazole) and C (buffer A + 300 mM imidazole) were used for equilibration, wash and elution respectively. The clear supernatant was filtered using a 0.45 µ syringe filter before loading onto the column. The filtered