ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 5, Issue 4, pp: (79-83), Month: October - December 2017, Available at: www.researchpublish.com

Auto-cleavage of GST Tagged Dengue Virus Type 2 NS2B-NS3 Protease

Sushil Kumar[1, 2, 3], Seema Dwivedi[2]

[1]Special Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi-110067, India
[2]School of Biotechnology, Gautam Buddha University, Greater Noida-201308, India
[3]Recombinant Gene Product group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi-110067, India

Abstract: **During the past five decades, dengue has evolved into one of the world's most widespread infectious diseases and now constitutes a major global public health problem. Four closely related dengue virus serotypes (DENV-1, -2, -3, -4), are the causative agents of dengue disease. The spread of dengue virus (DENVs) has been attributed to massive unplanned urbanization, overpopulation, increasing global travel, and the inability to eradicate mosquito vectors. Dengue virus RNA genome contains 10,723 nucleotides translating a single ORF that encodes polyprotein precursor of 3391 amino acids residues , following which are series of post- translational proteolytic processing events generate the mature viral products. The viral poly protein has three structural and 7 non-structural (NS) proteins: C (core), prM (precursor to membrane), E (envelope), NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. The multistep maturation process is helped by host proteases and dengue NS2B-NS3 protease by cleaving the viral polyprotein at certain sites into its constituent proteins. In this paper we report the self-cleavage activity of GST tagged NS2b-NS3 DENV type 2 protease.**

Keywords: **Auto-cleavage, dengue virus, protein purification, NS proteins.**

1. INTRODUCTION

Dengue virus **(**DENVs) are members of the *Flaviviridae* family and grouped within the flavivirus genus together with other pathogenic viruses including West Nile virus (WNV), Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), yellow fever virus (YFV), St. Louis encephalitis virus (SLEV) and Murray Valley encephalitis virus (MVLV) [1]. Dengue is the most rapidly spreading vector-borne viral disease in the world. In the last 50 years, incidence has increased more than 30-fold with increasing geographic expansion to new countries and, in the present decade, from urban to rural settings [2]. Around two billion people worldwide are at risk of dengue infection and ~100 million dengue infections are estimated to occur each year. Dengue infections result in around 25000 deaths each year, primarily in children [3]. Infection with DENV results in varying degrees of pathological conditions ranging from a mild febrile illness, dengue fever (DF), to severe and fatal dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) [4].

All flaviviruses including the DENVs share a similar genetic organization [5]. The flaviviral genome is ~11 kilobase (kb), 5' capped, poly-A tail lacking, single-stranded, (+) sense genomic RNA molecule of relatively simple organization. It has a single open reading frame (ORF), flanked by 5' and 3' untranslated regions (UTRs) of ~100 nucleotides (nts) and ~450 nts, respectively. These UTRs contain unique sequence and structural elements implicated in RNA-RNA and RNA-protein interactions, critical for the viral life cycle [4], [7]. This single ORF encodes a long polyprotein, which is processed by the combined action of viral and host proteases into three structural proteins, the C, M (which is synthesized

as the larger precursor, prM) and the E, and seven non-structural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 [5], [7], [8] .

2. MATERIALS AND METHODS

2.1 Cloning of NS2b-NS3 gene in pGEX4T3nn protein expression vector:

NS2b-NS3 gene was PCR amplified using respective forward (5'-CCG AAT TCT ACG TAA TGG CGG ATC TGG AAC TGG AAC TGG AAC-3') and reverse (5'-CCG AAT TCT ACG TAG AAG ATA TCA TCT TCG ATT TCC-3') primers. PCR product was purified and digested with *Sna*B1 restriction enzyme and 729 base pairs (bp) DNA fragment was cloned into *Sna*B1 digested pGEX4T3nn protein expression vector. Positive clones were screened by colony PCR and selected clone was checked for correct orientation of cloned gene by performing PCR with vector specific forward (5'-CAC GTT TGG TGG TGG CGA CCA TCC T-3') and gene specific reverse (5'-CCG AAT TCT ACG TAG AAG ATA TCA TCT TCG ATT TCC-3') primers. DNA was prepared and desired gene containing plasmid was authenticated by sequencing.

2.2 Expression and purification of GST-NS2b-NS3 protein:

NS2B-NS3 gene harbouring plasmid pGEX4T3nn was transformed with BL21 (DE3) strain of *E. coli.* Positive clones were screened by colony PCR. Expression of the N-terminal GST-tagged fusion protein was checked for several positive clones by inducing the culture with 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) at 37^{o}C for 3 hours in presence of ampicillin (100µg/mL). Clone 2 (Fig. 4a) showed the expression of the protein which was reflected by appearance of right size band (52.4 kDa) while running the whole cell lysate on SDS-PAGE stained with Coomassie Brilliant Blue R-250 stain .This clone was used for further processing . Protein expression profile was performed at three different temperatures, 37^{o}C (3 hours), 25^{o}C (6 hours) and 18^{o}C (16 hours). Maximum level of protein expression was achieved when induction was carried out at 18^{o}C for 16 hours which was confirmed by western blotting (Fig. 4b). Further, protein purification was carried out in which induced cell culture pellet was washed with PBS (Phosphate buffer saline), resuspended in PBS buffer and sonicated till a clear lysate was obtained. This lysate was then centrifuged at 10,000 rpm (Revolutions per minutes) for 1 hour, and the clear supernatant was added to a requisite amount of Glutathione Sepharose-4B beads equilibrated with PBS, and incubated at 4°C overnight with constant shaking. The column washed thrice with PBS. Resin bound protein was eluted using elution buffer (10 mM reduced glutathione in 50 mM Tris-Cl, pH 8.0).

2.3 Western Blotting:

Cell lysates of induced protein at different temperatures were run on SDS-PAGE and the protein bands were transferred onto nitrocellulose membrane. Membrane was blocked with 5% skimmed milk [in PBS (Phosphate buffer saline) containing 0.1% Tween 20] for two hours and washed with 0.1% PBST. The membrane immobilized protein bands were incubated with anti-GST antibodies (Sigma) (1:2000, in 2.5% skimmed milk in 0.1% PBST solution) for 1 hour and then again washed with 0.1% PBST. Finally, incubation with anti-mouse IgG HRPO-labeled secondary antibodies (Calbiochem) (1:5000) was carried out for 1 hour. Membrane was washed and the blot was developed using TMB (3, 3, 5, 5'-tetramethylbenzidine) substrate (Sigma).

3. RESULTS AND DISCUSSION

3.1 Design of NS2B-NS3 gene:

The DV NS2B-NS3 protease is a non- covalent complex of the NS2B and NS3 proteins. It is responsible for the cleavage at a number of sites on the viral polyprotein, including NS2A-NS2B, NS2B-NS3, NS3-NS4A and NS4B-NS5 [9]. NS3 protein contains a serine protease domain on N terminal. The minimum sequence that possesses protease activity has been attributed to the first 167 residues while the C terminal region of the NS3 contains conserved motifs that are found in several NTPases and RNA helicases [9]. Mutagenesis studies involving the NS2B protein have pointed to a central hydrophilic domain of approximately 40 amino acids within NS2B that is required for the optimal activity of NS3 protease [10]. In our study, 48 amino acids (aa) long NS2B gene was linked by G4-S-G4 (glycine$_4$-serine-glycine$_4$) to 180 aa long NS3 protease gene (Fig. 1).

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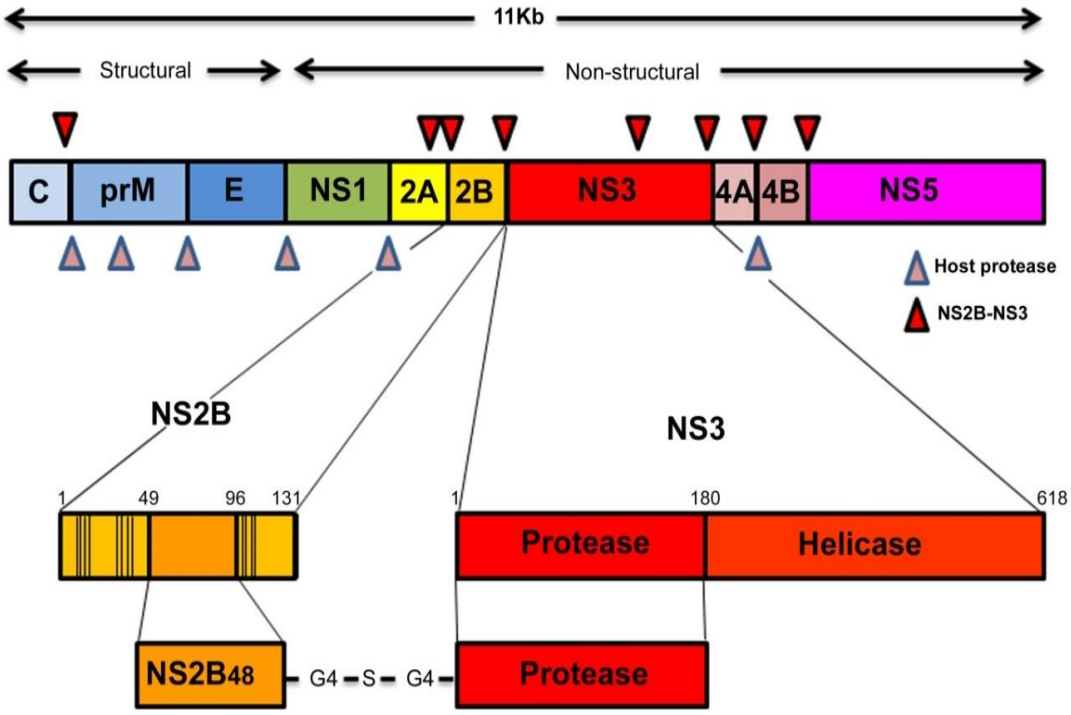

Fig. 1: Dengue virus genome and illustration of portion used to create chimeric NS2B-NS3 protein.

3.2 Creation of GST-NS2B-NS3 chimera:

Plasmid pGEX4T3nn carries the *Sna*B1 site for blunt-end cloning. The site immediately follows the end of the glutathione S-transferase (GST) gene and just precedes a stop codon. N terminal GST tagged NS2B-NS3 fusion protein assembly was created by cloning the NS2B-NS3 protease gene on *Sna*B1 restriction site in pGEX4T3nn plasmid vector, which resulted in the formation of NS2B-NS3pGEX4T3nn plasmid (Fig. 2).

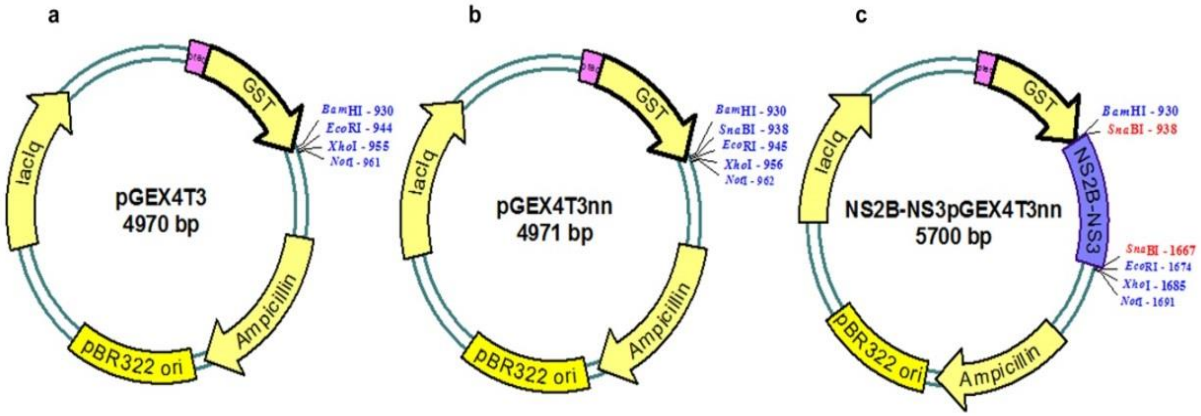

Fig. 2: Creation of GST-NS2B-NS3 chimera (a) Map of original pGEX4T3 vector plasmid. (b) pGEX4T3nn vector was created by inserting *Sna*B1 restriction site into original pGEX4T3 vector (c) NS2B-NS3 gene was cloned at *Sna*B1 site in pGEX4T3nn vector. pDRAW 32 DNA analysis software was used to generate above vector maps.

3.3 DENV GST-NS2B-NS3 auto-cleavage:

An integral tool for the purification of a chimeric protein is an affinity tag, with which a target protein can be obtained from a crude cell extract via step by step process [11]. It is necessary to know about the physical parameters such as molecular weight (MW), isoelectric point (pI) and hydrophobicity before checking the expression of a protein and purifying it. Protparam tool from the ExPASy server (http://web.expasy.org/protparam/) was used to ascertain these parameters for GST-NS2B-NS3 protein (Fig. 3).

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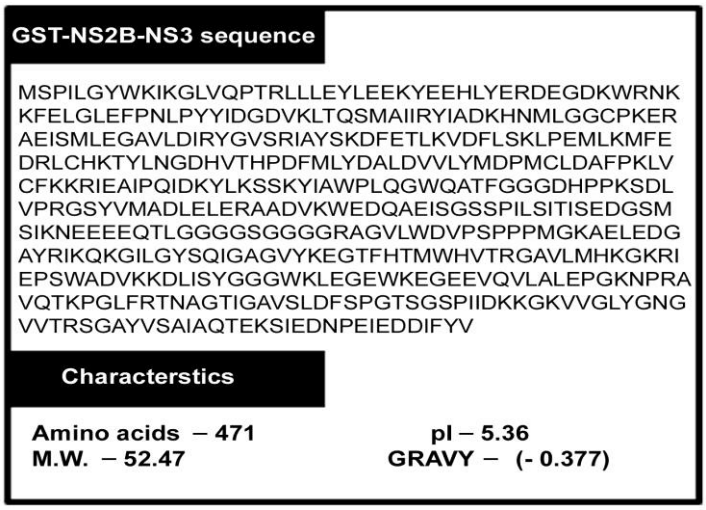

Fig. 3: Amino acid sequence of the GST-NS2B-NS3 protein. Protparam tool from the ExPASy server (http://web.expasy.org/protparam/) was used to ascertain MW, pI and hydrophobicity of GST-NS2B-NS3

The glutathione S-transferases (GST) are used to label proteins for expression and purification applications. Glutathione Sepharose-4B beads serve to bind the GST fusion protein, and gentle elution with reduced glutathione buffer. Enterokinase is generally used to cleave GST and the target protein [12]. Auto cleavage of protein was observed while checking the expression of GST-NS2B-NS3 protein at different temperatures, when we performed the western blotting experiment after running the whole cell lysate on SDS-PAGE (Fig. 4b).The auto cleavage of protein became more evident when we purified the protein. From figure 4(c) it is clearly reflected that being a serine protease NS2B-NS3 tend to cleave itself and its protein tag into different moiety during its expression and purification.

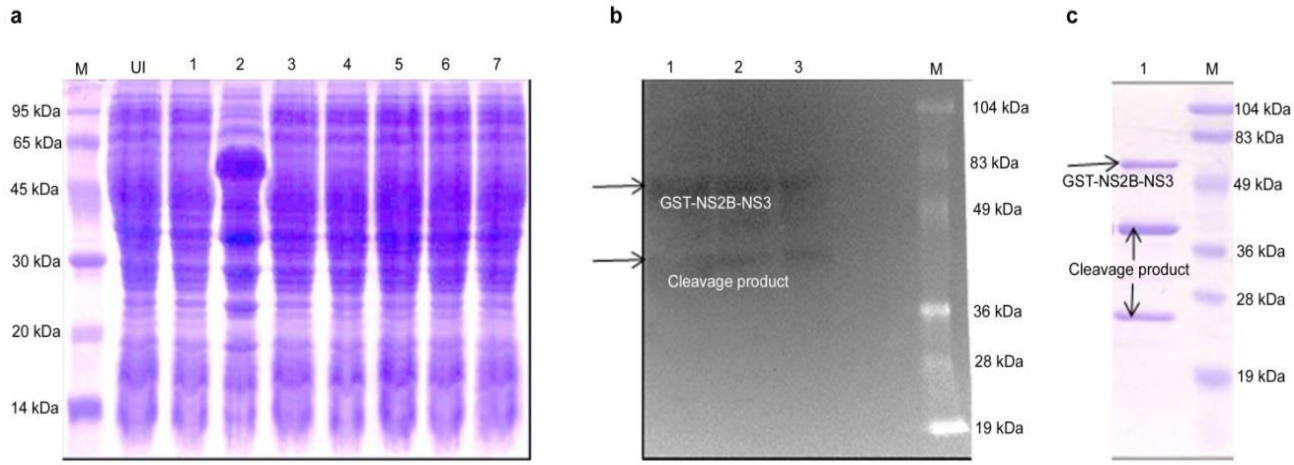

Fig. 4: Illustrating the auto cleavage of GST-NS2B-NS3 protein. (a) Expression of N-terminal GST-tagged fusion protein, GST-NS2B-NS3. Lane M: low molecular weight protein marker; lane UI: uninduced culture lysate; lane 1-7: induced culture lysate of different clones (1-7), clone 2 was used for protein purification (b) Western blot with anti-GST antibodies showing expression of GST-tagged NS2B-NS3 fusion protein induced at three different temperatures. Lane 1-3: protein expression induced at 18, 25 and 37^{o}C temperatures, respectively; at all temperatures protein tend to auto-cleave itself; lane M: prestained molecular weight protein marker. (c) Coomassie stained SDS-PAGE of purified GST-NS2B-NS3 protein. Lane 1: Pooled eluted fraction showing GST-NS2B-NS3 protein along with self-cleaved protein product, lane M: prestained molecular weight protein marker.

4. CONCLUSION

In summary, this study demonstrated the self-cleaving proteolytic ability of dengue virus type 2 NS2B-NS3protease during its purification with GST tag. Being a multifunctional protein, the dengue NS2B-NS3 protease is believed to play important role in host pathogen interaction. This study advocates about the nature of NS2B-NS3 protease, as its function is to process the viral polyprotein by cleaving it at different sites *in vivo*. Together with host proteases NS2B-NS3

protease serves as a responsible participant in viral maturation process of dengue. The linker G4-S-G4 ($glycine_4$-serine-$glycine_4$) might also have contributed to enhance the proteolytic ability of NS2B-NS3 protease, however, it need to be experimentally proven. Since, dengue virus belongs to the *Flaviviridae* family which also includes other pathogenic viruses; moreover, all flaviviruses share similar genetic organization. This study of the DENV NS2B-NS3 protease can pave the way to know the behaviour of the other flaviviruses proteases, especially which infect human such as HCV.

ACKNOWLEDGEMENTS

The authors of this research work would like to thanks Department of Biotechnology Government of India for financial support.

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