Overexpression of The Intact Membrane Integral Dengue Viral Protease Complex, NS2B-NS3

การสังเคราะห์โปรตีเอสเชิงซ้อน (NS2B-NS3) ของไวรัสไข้เลือดออกที่อยู่ในเยื่อหุ้มเซลล์

Opas Choksupmanee (โอภาส โชคทรัพย์มณี)* Dr. Gerd Katzenmeier (เกียด แคทเซนไมเออร์)**
Dr. Sarin Chimnaronk (ดร. ศรินทร์ ฉิมณรงค์)***

ABSTRACT

Dengue virus causes the dengue fever and dengue hemorrhagic fever/dengue shock syndrome, both of which at present lack effective drugs and vaccines for the clinical treatment. NS2B-NS3 protease complex is most attractive for drug development because of its essential roles in viral RNA replication. As this protein complex is an intrinsic membrane integral protein, many critical problems such as protein misfolding and the low yield protein production emerge during the protein expression trial. Here we report the use of a small membrane protein, Mistic, to address the problem. The newly constructed expression pMHTD vector with the Mistic fusion revealed the first successful case of the high-level protein expression of the dengue protease complex in *Escherichia coli* cells.

บทคัดย่อ

ไวรัสไข้เลือดเป็นสาเหตุของโรคไข้เลือดออก ซึ่งปัจจุบันยังไม่มียาและวัคซีนที่มีประสิทธิภาพในการรักษาโรค โปรตีเอสเชิงซ้อน NS2B-NS3pro จึงเป็นเป้าหมายในการพัฒนายาและวัคซีน เนื่องจากโปรตีนชนิดนี้มีส่วนเกี่ยวข้องกับการเพิ่มปริมาณของไวรัส โดยโปรตีเอสเชิงซ้อน NS2B-NS3pro เป็นโปรตีนที่แทรกตัวอยู่ในเยื่อหุ้มเซลล์ การสังเคราะห์โปรตีนจึงมีปัญหาเกิดขึ้น คือ โปรตีนมีการจัดเรียงตัวไม่ถูกต้องและมีปริมาณน้อย งานวิจัยชิ้นนี้จึงได้เลือกใช้เวคเตอร์ pMHTD ที่มีโปรตีนมิสทิค (Mistic) ในการแก้ปัญหา ซึ่งจากผลการทดลองแสดงให้เห็นว่า NS2B-NS3pro ถูกสังเคราะห์ขึ้นในปริมาณที่สูงมากเมื่อเปรียบเทียบกับเวคเตอร์ชนิดอื่น

Key Words : NS2B-NS3 protease, Mistic

คำสำคัญ : โปรตีเอสเชิงซ้อน NS2B-NS3 โปรตีนมิสทิค

**Master Student, Master of Molecular Genetics and Genetic Engineering program, Institute of Molecular Bioscience, Mahidol University*

***Assistant Professor, Institute of Molecular Bioscience, Mahidol University*

****Lecturer, Institute of Molecular Bioscience, Mahidol University*

Introduction

Dengue virus (DV) is a member of the Flaviviridae family that is transmitted to human via mosquitoes such as *Aedes aegypti*. Dengue is a major public health problem, with about 50 million people infected each year (of who about 20,000 die) and about 2.5 billion people worldwide being at risk of infection. Dengue fever is characterized by high fever, chills, body aches, and skin rash and ranges from mild, flu-like symptoms to severe forms, which are dengue hemorrhagic fever and the life threatening hypovolaemic dengue shock syndrome. Unfortunately, no DV-specific therapies or vaccines are available at present. (Henchal et al 1990, Perera et al 2008).

DV is the positive stranded RNA of 11 kb which encodes for a single polyprotein that consists of three structural (C, prM and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). For a new viral production, this polyprotein is translocated into the endoplasmic reticulum (ER), and specifically processed by host signalases and the viral NS2B-NS3 protease to be mature and active (Leung et al 2001).

NS3 is a multi-domain protein of 69 kDa containing prortease domain at N terminus. The protease domain form a chymotrypsin-like serine protease structure interacting with the other functions of NS3 (RNA helicase and RTPase/NTPase), NS2B, and NS5 for viral RNA replication. This domain consists of six β strands forming two β barrels with His-51, Asp-75 and Ser-135 catalytic triad. In term of protease activity of NS3, it depends on a small activating protein, NS2B (14 kDa) that has abundantly conserving hydrophobic amino acid among flaviviruses but the structure of NS2B is still mysterious. Due to NS2B-NS3 protease complex is essential for viral maturation (viral protein processing) thus many researchers play attention to study this protein complex. But several papers concentrated only the role of hydrophilic part of NS2B interacting with NS3 protease (Leung et al 2001, Niyomrattanakit et al 2004 and Roosild et al 2005).

In the other hand, very little is known about the exact mechanism of the full-length NS2B and NS3 protease. Therefore this study aims to construct the NS2B-NS3 protease complex for further study in the structure and mechanism.

Difficulty in obtaining the structural information of NS2B derives from the fact that NS2B is a membrane-associated protein. It is proposed that NS2B contains three to four transmembrane helices with a flexible cytoplasmic loop where NS3 protease binds, as predicted by the hydrophobic score from the amino acid sequence. Attempt to overexpress the full-length NS2B (14 kDa) and NS3pro (20 kDa) in *Escherichia coli* (*E. coli*) yielded the protease in an insoluble form, and required denaturing/refolding process to purify the protein. The refolding step dramatically dropped the yield of the purified protein, and may not guarantee the folding property of the protein as in the membrane environment *in vivo*. Thus, there is a requirement for a new expression system that is suitable for overexpression of NS2B-NS3pro in a membrane-associated form, which will allow the measurement of the protease activity in the membrane environment and also provide an avenue

for the structural study of the 'real' dengue protease complex.

Materials and methods

1. Construction of the expression vector

The construction of the expression vector was divided into two steps; the introduction of the Mistic protein following by an octahistidine tag (8xHis) and a thrombin cleavage site (MHT fragment), and the cloning of the dengue NS2B-NS3 protease (NS2B-NS3pro), into pETDuet-1 vector (Novagen). To create the MHT fragment, the *Mistic* gene from *Bacillus subtilis* was amplified by two specific primers, MistFw (5'-CATGACCATGGGCTTTTGTACATTTTTTG AAAAACATC-3') and MistRV1R (5'-GGTGAT GATGTCCAGAACCTCCAGAGCCTTCTTTTTC TCCTTCTTCAGATAC-3'). This fragment is designed as F1. Subsequently, the rest fragment F2 containing a His-tag and the thrombin cleavage site were produced by PCR with two overlapping primers, MistRV2F (5'-GGTTCTGGACATCATCAC CATCATCATCACCACGGTTCTAGCGGATTAG-3') and MistRV3R (5'-TGACGGATCCTGGCTAGATCC GCGTGGCACTAATCCGCTAGAACCGTGG-3'). Finally, F1 and F2 fragments were connected by PCR ligation with an overlapping sequence on 3′ and 5′-end of F1 and F2, respectively. The MHT fragment was inserted between *Nco*I and *Bam*HI sites preceding the multi-cloning site 1 (MCS1) in pET-Duet-1 (**Figure 1**). The resulting plasmid, pMHTD, was verified by DNA sequencing of 5 selected positive clones.

Cloning of the NS2B-NS3pro from the dengue virus serotype 2 was performed by PCR of pDHMT vector with two specific primers, NS2B3FW (5'-GATCGAATTCGTGGCCATTAA ATGAGGCTATCATG-3') and NS2B3PRV (5'-ATCGAAGCTTATCGGAAAATGTCATCTTCGA TCTC-3'), and was inserted into pMHTD using *Eco*RI and *Hind*III in MCS1. The full-length of the recombinant was confirmed by DNA sequencing.

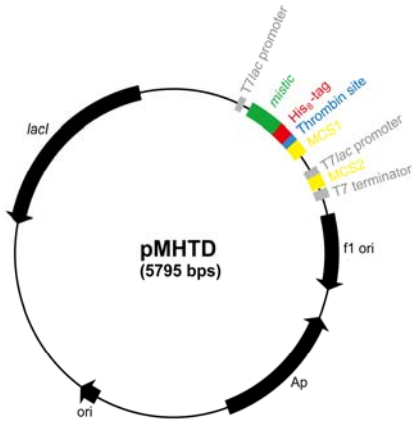

Figure 1. pMHTD expression vector for dengue NS2B- NS3 protease complex.

2. Protein expression

The recombinant clone was transformed into BL21 (DE3) + pRARE2 competent cells using the heat shock method. A single colony carrying the recombinant plasmid was grown in LB broth containing 100 µg/ml amplicillin, 50 µg/ml chloramphenicol and 1% D-glucose as a starter cells at 37°C for overnight. Later on 1% of starter cells were inoculated into the same fresh medium. The cultures were incubated at 37°C until OD_{600} reached to about 0.6. Then 100 µM of IPTG was added and additionally incubated at 25°C for 6 hrs. The cultures were harvested by centrifugation at 6,000g for 10 min (TOMY MX-30). Subsequently the cells were lysed by sonication on ice with the lysis buffer

containing 50 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 10% glycerol, 250 mM NaCl, 7 mM β-mercaptoethanol, 0.5 mg/ml lysozyme, and 1mM PMSF. The cell lysates were fractionated by centrifugation at 100,000 g [Beckman Optima L-70 K ultracentrifuge (Beckman Coulter, Inc., Fullerton, Calif.)] for 1 hr to obtain soluble and insoluble fractions.

3. Extraction of membrane protein

Detergent screening was achieved by adding 1-2% of various detergents (**Table 1**) into the insoluble fraction of the proteins, and gentle mixing for overnight at 4°C. The solubilized protein fractions were collected by the ultracentrifugation at 100,000g for 1 hr at 4°C. The solubilized proteins were analyzed by 12% SDS-PAGE.

Table 1 List of detergents using for membrain protein solubilization

List of the detergents

	Detergent	Type	Conc	CMC
1	Triton X-100	non-ionic	1%	0.0150%
2	Tween 20	non-ionic	1%	0.0072%
3	Tergitol NP-10	non-ionic	1%	0.0040%
4	LDAO	non-ionic	1%	0.0230%
5	n-Dodecyl-β-D-maltoside	non-ionic	1%	0.0087%
6	n-decyl-β-D-maltoside	non-ionic	1%	0.0870%
7	n-Undecyl-β-D-maltoside	non-ionic	1%	0.0290%
8	n-Octyl-β-D-maltoside	non-ionic	2%	0.5300%
9	n-Nonyl-β-D-glucoside	non-ionic	2%	0.2000%
10	Sucrose monolaurate	non-ionic	1%	0.0100%
11	C12E8	non-ionic	1%	0.0059%
12	CTAB	cationic	1%	0.0360%
13	Sodium cholate	anionic	2%	0.4100%
14	CHAPS	Zwitterion-ionic	2%	0.4900%
15	Fos-choline-10	Zwitterion-ionic	1%	0.35%
16	Fos-choline-12	Zwitterion-ionic	1%	0.0470%
17	Fos-choline-14	Zwitterion-ionic	1%	0.0051%
18	None (MilliQ)	-	-	-

4. Western blot analysis

The NS2B-NS3pro complex was separated on 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 1% skim milk in PBS containing tween-20 for 1 hr at room temperature, and incubated at room temperature for 15 min with anti-His-tag-HRP (1:1000) antibodies. To visualize the bands, the membrane was incubated with chemiluminescent HRP substrate for 1 min at room temperature.

Results and discussion

There are so far no any reports showing the successful expression and purification of the full-length NS2-NS3pro. The difficulty in overexpression of the NS2B-NS3pro complex is ascribed to the fact that NS2B is an intrinsic membrane protein. Using a classical expression vector pET yielded only very low amount of protein expression, and the expressed protein was thought to be misfolded, thus forming the aggregation or the inclusion body in *E. coli* cells (**Figure 4A, lane 9**). To overcome the problem, we created a unique vector for the heterogeneous expression of the membrane protein in *E. coli*, called pMHTD (**Figure 2**). We take an advantage of the Mistic protein that autonomously folds into the *E. coli* membrane independent of the sorting machinery. We exploited Mistic as a tag fused to the N-terminus of the target membrane protein, with an expectation that Mistic would help NS2B to insert and fold properly into the membrane, and thus, escape the aggregation and degradation in *E. coli* cells.

To create pMHTD vector, Misitc and the His-tag were separately amplified and subsequently ligated together using PCR. PCR products were analysed on a 5% native acrylamide gel (**Figure 2**). As there were small traces of both F1 (368 bp) and F2 (428 bp) fragments after PCR ligation, the MHT fragment was further purified by the gel extraction kit (QiaGen) prior to the insertion into pETDuet-1 vector (**figure 3**). We put the MHT immediately upstream of MCS1, allowing to clone any membrane protein genes downstream. Moreover. it is also possible to clone any ligand proteins that interact with the target membrane protein into the vacant MCS2 to co-express in *E. coli*. This universal membrane protein expression vector (5795 bps) was designated "pMHTD".

Afterward the full-length den2 NS2B-NS3pro was cloned into pMHTD, and the protein expression was examined in a small scale. The result showed that NS2B-NS3pro was extremely expressed in a high yield in the presence of the Mistic tag compared to that of the pET-28a control (**Figure 4**). This is the first success reporting on the expression of the full-length dengue NS2B-NS3pro complex.

As it was expected that NS2B would properly fold into the membrane with the help of Mistic, the detergent was used for the extraction of the membrane protein. We tested seventeen detergents with NS2B-NS3pro (**Table 1** and **Figure 5**).

The result reveled that LDAO, CTAB, and Fos-choline were suitable for the extraction and solubilization of NS2B-NS3pro from the *E. coli* membrane. Fos-choline 14 gave the best yield and this detergent was chose for the further experiments. Note that LDAO was also used as a detergent to stabilize Mistic in the original report (Roosild et al 2005).

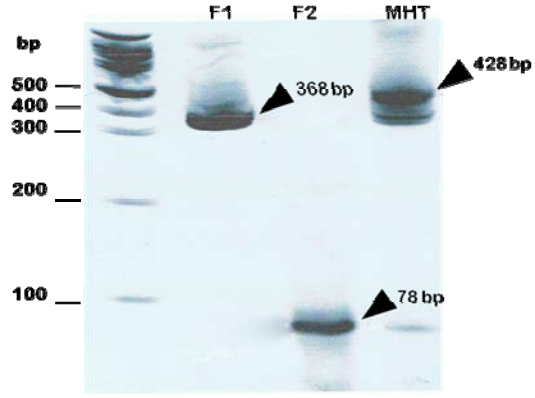

Figure 2 The synthesis of the MHT fragment. PCR products were analyzed by 5% native poly-acrylamide gel electrophoresis. The size of F1 and F2 fragments were 368 bps and 78 bps, respectively. When these fragments were connected using PCR ligation to produce MHT fragment, its size became 428 bps.

For further chemical and structural characterization studies, after successfully solubilizing NS2B-NS3pro by Fos-choline-14, we need to remove the Mistic tag from the recombinant NS2B-NS3pro by the thrombin treatment. Subsequently, NS2B-NS3pro and Mistic tag, will be confirmed by western blot analysis with anti-His and anti-NS3pro antibodies.

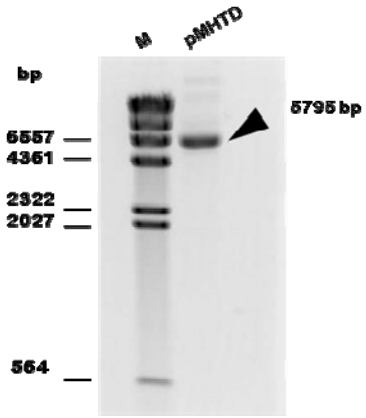

Figure 3. pMHTD expression vector (lane 1; λ/HindIII marker, lane 2; pMHTD).

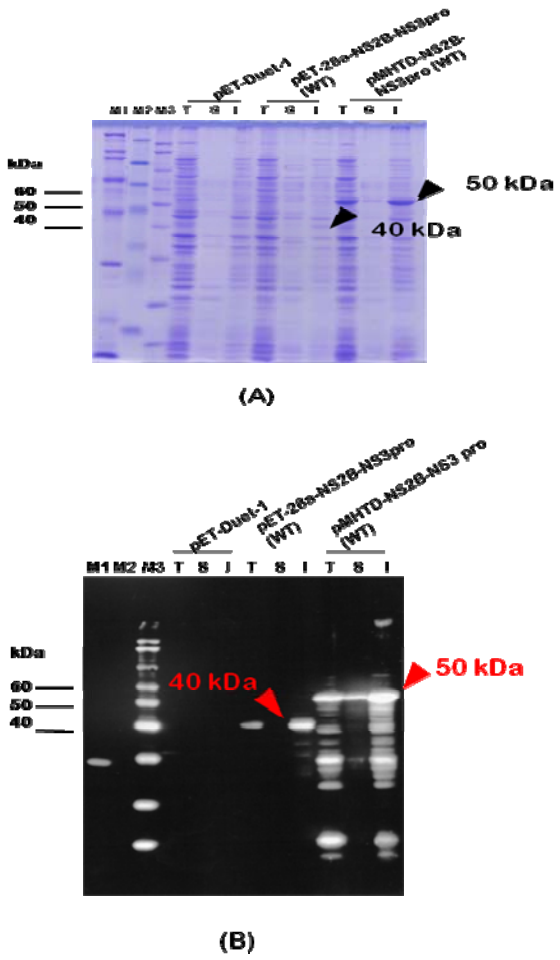

Figure 4 Expression analysis of NS2B-NS3pro. (M1, broad range marker; M2, prestained protein marker; M3, BenchMark™ protein ladder; T, total protein; S, soluble fraction; I, insoluble fraction). (A) pMHTD and pET-28a express-ion vectors were used to express NS2B-NS3pro with and without Mistic tag, respec-tively. The size of Mistic-tagged and non-tagged NS2N-NS3pros are approximately 50 and 40 kDa, respectively. (B) The result was further evaluated by western blot analysis using anti-His (1:1000)

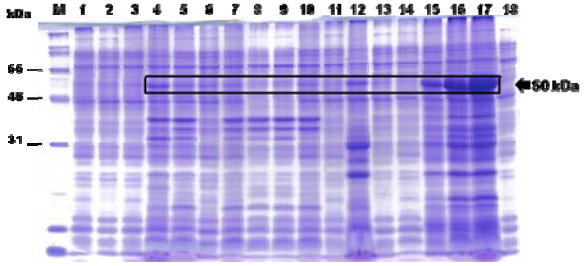

Figure 5 Detergent screening for membrane protein solubilization. 12% SDS-PAGE analysis was used to detected the effect of various deter-gents in the solubilization of NS2B-NS3pro. LDAO (lane 4), CTAB (lane 12), Fos-choline -10 (lane 15), Fos-choline-12 (lane 16) and Fos-choline-14 (lane 17) can solubilize NS2B-NS3pro.

Conclusions

The dengue NS2B-NS3pro was, for the first time, successfully expressed in a high yield as a membrane integral protein in *E. coli* using the new constructed pMHTD expression vector. Moreover we found that Fos-choline-14 is the best detergent to solubilize NS2B-NS3pro complex.

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