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การผลิตโปรตีนเอนวิโลปสายสั้นจากเชื้อไวรัสเดงกี่และความสามารถในการกระตุ้นภูมิคุ้มกันในหนู BALB/c

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Production of Truncated Recombinant Dengue Virus Envelope Protein and its Immunogenicity in BALB/c Mice

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หลักการและวัตถุประสงค์: โรคไข้เลือดออกเดงกี่เป็นปัญหาทางสาธารณสุขที่สำคัญในประเทศเขตร้อนและกึ่งร้อนชื้น เชื้อไวรัสเดงกี่ทั้ง 4 สายพันธุ์ก่อให้เกิดโรคที่มีอาการรุนแรงแตกต่างกันไป ในปัจจุบันยังคงมีการวิจัยและพัฒนาวัคซีน ยารักษา และชุดตรวจวินิจฉัยให้มีประสิทธิภาพที่ดีขึ้นอย่างต่อเนื่อง โปรตีนเอนวิโลป (envelope protein; E) เป็นโปรตีนเป้าหมายหลักของการตรวจจับโดยแอนติบอดีและเป็นสารกระตุ้นภูมิคุ้มกันที่ดี วัตถุประสงค์ในการศึกษาวิจัย คือ ผลิตโปรตีน rE74-118 จากสารพันธุกรรมของเชื้อไวรัสเดงกี่สายพันธุ์ที่ 2 และศึกษาคุณสมบัติเบื้องต้นของโปรตีนทั้งในหลอดทดลองและสัตว์ทดลอง

วิธีและผลการศึกษา: ผลิตโปรตีน rE74-118 ด้วยวิธีการโคลนนิ่งใส่พลาสมิด pET-32b และแสดงออกโปรตีนในเชื้อแบคทีเรีย *Escherichia coli* (SHuffle) หลังจากนั้นทำโปรตีนให้บริสุทธิ์ ได้ความเข้มข้นของโปรตีน 5.8 มิลลิกรัมต่อมิลลิลิตร ทำการทดสอบคุณสมบัติของโปรตีนในการจับจำเพาะกับโมโนโคลนอลแอนติบอดีและซีรั่มของผู้ป่วยโรคไข้เลือดออกเดงกี่ และนำไปทดสอบความสามารถในการกระตุ้นภูมิคุ้มกันในหนู BALB/c พบว่าโปรตีน rE74-118 สามารถจับกับโมโนโคลนอลแอนติบอดีและซีรัมของผู้ป่วยได้อย่างจำเพาะ และสามารถกระตุ้นภูมิคุ้มกันแบบสารน้ำในการต้านเชื้อไวรัสเดงกี่สายพันธุ์

Background and objective: Dengue is a major public health problem in tropical and subtropical countries. The four dengue virus serotypes can cause a wide range of mild to severe diseases. Many research and development efforts are ongoing to find a better effective and accessible version of vaccine, therapeutic agent, or diagnostic tool. Envelope (E) protein is a primary target for serologic diagnosis and immunization. This work aimed to express and characterize truncated E (rE74-118) protein of dengue virus serotype 2 (DENV-2) in both *in vitro* and *in vivo* properties.

Methods and results: A truncated DENV-2 envelope E protein, amino acid sequence 74-118, encoding gene was amplified and cloned into the pET-32b plasmid. The recombinant plasmid was then expressed in *Escherichia coli* (SHuffle) to produce the recombinant protein rE74-118. Recombinant E protein in truncated form (rE74-118) was successfully constructed, expressed, and purified in the concentration of 5.8 mg/ml. The rE74-118 protein was tested for its

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ที่ 2 ได้ในระดับอ่อนๆ และไม่เพิ่มความไวในการติดเชื้อ
สรุป: โปรตีน rE74-118 มีคุณสมบัติในการจับจำเพาะ (antigenicity) และมีความสามารถในการกระตุ้นภูมิคุ้มกัน (immunogenicity)

คำสำคัญ: ไวรัสเดงกี่; โปรตีนเอนวิโลป; แอนติเจน; อิมมิวโนเจน

specificity with an anti-E monoclonal antibodies and dengue patient sera. Furthermore, its immunogenicity in BALB/c mice was also tested. The results showed that the rE74-118 protein can specifically react to anti-E monoclonal antibodies and dengue patient sera. This protein also induces the humoral response with a low-level of neutralizing activity against DENV-2, as well as the protein do not show enhancing activities against all four serotypes.
Conclusion: The truncated rE74-118 protein showed both antigenic and immunogenic properties in these *in vitro* and *in vivo* characterizations.

Keywords: Dengue virus; truncated envelope protein; antigen; immunogen

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Introduction

Dengue is a major public health problem in tropical and subtropical countries, where the rainy season coincides with high temperatures. Dengue fever, dengue hemorrhagic fever, or dengue shock syndrome is caused by one of four serotypes of dengue virus (DENV) in the Flaviviridae family, which is transmitted to human by the infected female *Aedes aegypti* or *Aedes albopictus* mosquitoes. Secondary infection with heterotypic serotypes from primary infection leads to severe dengue scenario, which is induced by enhancing activity of pre-existing antibody response against the remaining three serotypes, known as antibody dependent enhancement[1]. DENV-2 show the lowest efficacy in dengue vaccine. Envelope (E) protein is composed of three structurally and functionally different domains including domain I (EDI), domain II (EDII), and domain III (EDIII)[2]. Previous studies demonstrated that the most potently and broadly neutralizing epitopes of human monoclonal antibodies are on EDII region, which is one of several antigens that could be potentially designed for using in diagnostic and therapeutic applications[3, 4-6]. Expression and characterization of short and stable forms of truncated dengue EDII protein is still limited[7,8]. This rE74-118 construct was developed with minimum binding region. We hope to see specific binding, some neutralizing and low enhancing activities.

In this study, we aimed to produce and characterize a short recombinant E74-118 (rE74-118) protein encoded from dengue virus serotype 2 envelope (DENV-2 E) gene. The encoding gene was cloned into a pET-32b plasmid containing histidine tag and subsequently expressed in the *E. coli* (SHuffle). Then, we tested the binding reactivity of the rE74-118 with anti-E human monoclonal antibodies (huMAbs) and dengue patient sera by immunofluorescent assay. The purified rE74-118 was also tested its immunogenicity by subcutaneous injecting into the BALB/c mice. We expected that this rE74-118 could show both antigenic and immunogenic properties in these *in vitro* and *in vivo* characterizations. The rE74-118 might be a useful target for broad spectrum inhibitors, subunit vaccine candidates or economical screening tools for dengue infection.

Materials and Method

Construction of DENV-2 E74-118 gene in the pET-32b plasmid

The amplification of DENV-2 (NGC strain) E gene was done by using DV2_Env-74_f (5'-TGC CCA ACA CAA GGA GAA CCC AG-3') primer, DV2_Env-118_r (5'-CAT AGC ACA GGT CAC AAT GCC TC-3') primer, and KOD-Plus-Neo DNA polymerase (Toyobo, Japan) in order to produce E74-118 gene. The PCR reaction was performed at 98 °C for 2 min followed by 35 cycles of 94 °C for 10 sec, 55 °C for 30 sec, and 68 °C for 10 min. This gene was double digested and ligated into the pET-32b vector by using *Bam*HI, *Xho*I restriction enzymes (NEB, USA), and T4 DNA ligase (Thermo Scientific, Sweden). The inserted plasmid was transformed into *E. coli* (SHuffle). The transformants

were selected by ampicillin and confirmed by colony PCR using pET-32b_f (5'-GGG TGC ACT GTC TAA AGG TC-3') and pET-32b_r (5'-CAC CGC TGA GCA ATA ACT AG-3'). The correction of inserted gene was evaluated by DNA sequencing with the sequencing primers of pET-32b_f and pET-32b_r primers.

Expression and purification of DENV-2 rE74-118 protein in E. coli (SHuffle)

Positive clones were cultured in 200 ml medium at 25 ℃ for 20 hours with shaking and induced with 0.1 mM final concentration of Isopropyl-B-D-thiogalactopyranoside (IPTG; Thermo Scientific, USA). The cells were centrifuged at 8,000×g for 10 min. The pellet was re-suspended in four volumes of lysis buffer (20 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 M NaCl, 10% Glycerol, 0.2 mg/ml lysozyme, 0.2% Triton-X), and later sonicated at 10 Hz for 3 min with 30 s pulse. The sonicated cells were centrifuged at 10,000×g for 40 min and the supernatant was passed through Ni-NTA agarose column (Thermo Scientific, USA). The column was washed with 5 column volume of wash buffer (5 mM imidazole in lysis buffer) and eluted with 10 column volume of elution buffer (200 mM imidazole in lysis buffer). The eluted fractions were dialyzed against PBS to remove the traces of imidazole.

SDS-PAGE and western blot analysis of rE74-118 protein

The purified protein (100 ng) were loaded into each well of 15% acrylamide separating gel, then stained with Coomassie brilliant blue dye.The similar loading proteins were blotted onto the polyvinylidene difluoride (PVDF) membrane and incubated with 2 µg/ml of anti-dengue E huMAbs and HRP conjugated goat anti-human IgG (1:5,000). The immunoreactive bands were visualized under a chemiluminescence camera model LAS 4,000 mini (GE Bioscience).

Mouse immunization and blood collection

Six-week-old female BALB/c mice (25-30 g body weight) were purchased from Nomura Siam International Company, Bangkok, Thailand and housed in the animal facilities at the Faculty of Tropical Medicine. This study was approved by the Faculty of Tropical Medicine Animal Care and Use Committee (FTM-ACUC), Mahidol University with approval number FTM-ACUC 001/2014. Six mice were subcutaneous injected with 100 µg/dose of purified rE74-118 (n = 2), inactivated DENV-2 (NGC) (n=2), or PBS (n=2) combined with the Gerbu adjuvant (Nacalai tesque, Japan) (ratio = 1:1, volume = 100 µl/dose, 3 doses with 2 weeks apart). Blood was collected from retro-orbital sinus (0.1 - 0.2 ml/mouse) at day 0, 14, 28, and 42 for testing the level of specific, neutralizing, and enhancing antibodies.

Enzyme-linked immunosorbent assay (ELISA) for dengue IgG titers

Microtiter plate was coated with each DENV (DENV-1 to DENV-4) overnight at 4 ℃, washed with PBS containing 0.05% Tween-20 (PBS-T) for 3 times, and blocked with 200 µl/well of 1% BSA in PBS-T for 2 hour at 37 ℃. After three washes, the plate was added with 100 µl of mouse serum (diluted 1:100, 1:200, ..., 1:204,800), incubated for 1 hour at 37 ℃, washed for 3 times, added with 100 µl/well of HRP conjugated goat anti-mouse IgG (1:10,000 in PBS-T), and incubated for 1 hour at 37°C. After three additional washes, each well was added 100 µl of TMB substrate (Thermo Scientific, Sweden) and stopped with 25 µl of 2 N sulfuric acid. The absorbance was measured at OD 450 nm under the ELISA reader (Tecan®, Switzerland).

Focus reduction neutralization test (FRNT) in Vero cell

Heat inactivated mouse sera were serially diluted 2 folds (1:20, 1:40, 1:80, ... , 1:20,480) in serum-free medium, mixed with each DENV (DENV-1 to DENV-4; 100 FFU/well), and incubated at 37 ℃ for 1 hour. Each mixture was inoculated onto Vero cell in 96-well plate, incubated at 37°C for 1 hour, added 100 µl of 2% carboxymethyl cellulose (CMC) medium (supplemented with 3% FBS), and then incubated for 2-3 days. After that, cells were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, and stained with anti-dengue E huMAbs and anti-human-ALEXA 488 (1:500). The fluorescent signals on infected cells were observed under fluorescence microscope (IX71, Olympus). The neutralizing activities were calculated as 50% focus reduction neutralization test ($FRNT_{50}$).

Antibody dependent enhancement (ADE) assay using K562 cells

The serially diluted mouse sera (1:20, 1:40, 1:80, ... , 1:20,480) were incubated with each DENV (1 infected cell/well) for 30 minutes at 37 ℃. Then, K562

cells were treated with serum-virus mixture, incubated at 37 °C for 1 hour, added with 2% CMC medium (supplemented with 3% FBS). After incubation at 37 °C for 2-3 days, the cells were fixed with 3.7% formaldehyde and permeabilized with 1% Triton X-100. The infected cells were probed with anti-dengue E huMAbs and anti-human-ALEXA 488 (1:250). Immunostaining in each well was observed under fluorescence microscope (IX71, Olympus).

Results

Construction, expression, and purification of rE74-118 protein

Truncated rE74-118 gene was successfully cloned into pET-32b plasmid between the *Bam*HI and *Xho*I restriction sites located before histidine tag sequence at the C' terminal as shown in Figure 1A. The correction of inserted gene was confirmed by sequencing and double digestion (data not shown). To maximize the soluble protein yield of this construct , we optimize expression (37 °C for 2-4 hours, 30 °C for 4-6 hours, 22-25 °C for 6-20 hours and 12-15 °C overnight using 0.1 mM IPTG) and found that the best condition to express rE74-118 in *E. coli* (SHuffle) system is the induction with 0.1 mM IPTG, at 25 °C, for 20 hours (data not shown). The expected size of expressed protein is approximately 17 kDa with small amount of insoluble part and high amount of soluble part as shown in figure 1B. After that, we purified this protein by using His-Tag protein purification column and demonstrated each protein fraction with anti-E huMAbs staining as shown in figure 1C. The concentration of purified protein in elute 1 and 2 fractions were calculated by Bradford assay and the total concentration is 5.8 mg/ml obtained from 200 ml culture.

In vitro binding activity of rE74-118 protein

Expressed rE74-118 protein was tested for specific binding activity with anti-dengue E huMAbs, anti-dengue prM, anti-dengue NS1, and dengue patient sera in acute phase of DENV-2 infection as shown in figure 2. D23-1B3B9, D23-1G7C2, D23-1C2D2 anti-E huMAbs and dengue patient sera (D22, D25, D26, D27, D28, D29, D30, D32 and D33) showed strong reactivity with rE74-118. D22-2G4E3, D30-3B10E7 anti-E huMAbs and D23 patient serum showed weak binding activity. However, this rE74-118 did not bind with anti-dengue E huMAbs (D30-1E7B8, D32-2D1G5, D33-3E4H10, D23-1A10H7) and anti-dengue prM (D25-4D4F10) and anti-dengue NS1 huMAb (D26-5A2B12).

Immunogenicity of rE74-118 protein in BALB/c mice

Purified rE74-118 protein was tested *in vivo* activity by injecting 100 µg of rE74-118 combined with Gerbu adjuvant (ratio = 1:1) into the BALB/c mice for 3 doses (2 weeks apart) and it can induce dengue IgG antibodies after second boosting with the titer of 1:204,800 similar to whole DENV-2 immunization, and PBS with Gerbu regimen could not induce any dengue IgG antibody (data not shown). Neutralizing activity of

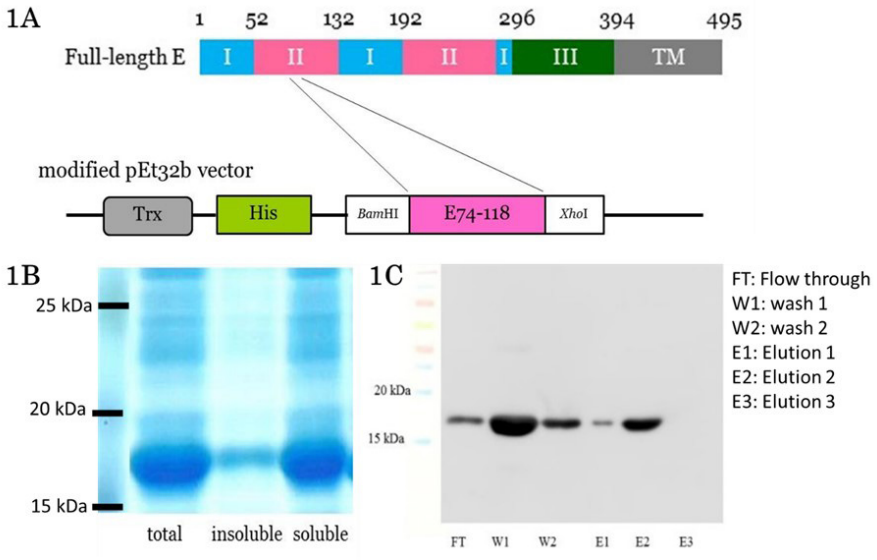

Figure 1A Map of rE74-118 in pET-32b plasmid
Figure 1B SDS-PAGE of rE74-118 protein
Figure 1C Western analysis of purified rE74-118 protein

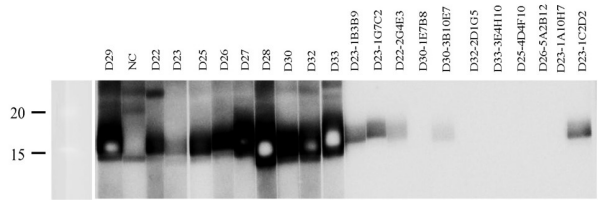

Figure 2 Binding activity of rE74-118 protein with dengue patient sera, anti-dengue E, anti-dengue prM, anti-NS1 huMAbs.

sera immunized with rE74-118 was detected in Vero cell and calculated as FRNT50 around 1:16 (data not shown). By ADE assay using K562 cell, mouse sera immunized with DENV-2 showed enhancing activities against all 4 serotypes, but no enhancing activity of mouse sera immunized with rE74-118 with any serotype as shown in figure 3.

Discussion

DENV E protein is one of several antigens could be designed for vaccine, inhibitor, or diagnostic agent. It can induce both of protective and pathological effects against DENV infection[8]. The expression and well-characterization of truncated E protein is still limited. This study aimed to express and characterize a short E protein, rE74-118, and its binding, neutralizing, and enhancing activities.

We successfully expressed rE74-118 in soluble form by using *E. coli* (SHuffle) with the high amount purified protein of 5.8 mg/ml from 200 ml bacterial culture and showed basic functions (e.g. binding activities, some neutralizing activities, and no ADE) similar to other expression system[9]. For binding activity, the short rE74-118 protein showed strong binding to dengue patient sera and most of anti-dengue E huMAbs, but no reactivity to anti-dengue prM huMAbs, and anti-dengue NS1 huMAbs, which is good for diagnostic application. Several anti-dengue E huMAbs (D30-1E7B8, D32-2D1G5, D33-3E4H10, D23-1A10H7) could not bind to rE74-118 because these antibodies might bind to the conformational epitopes[4, 10, 11]. Purified rE74-118 protein can stimulate humoral immune response in BALB/c mice due to the evidence that we can detect high amount of dengue IgG in mouse sera. The protective activity of rE74-118 was also investigated in our present study. We found that sera from mice immunized with rE74-118 show the low neutralizing activity against DENV-2, but no ADE activity with any serotype, which is better than the whole DENV-2 immunogen.

In conclusion, our rE74-118 protein showed both antigenic and immunogenic properties, that might be a promising target for various applications such as subunit vaccine, biological inhibitor, or diagnostic applications[4, 6, 8].

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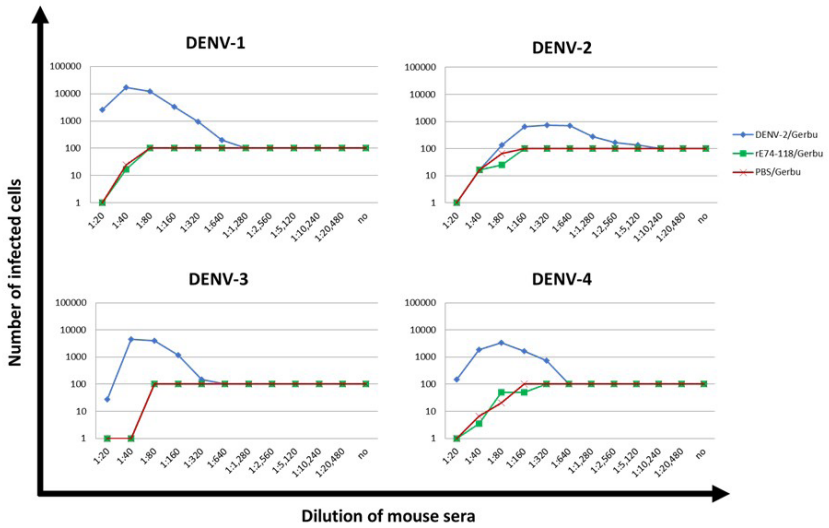

Figure 3 ADE activity of mouse sera against 4 serotypes of DENV

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