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Selection of suitable detergents for obtaining an active dengue protease in its natural form from *E. coli*

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ABSTRACT

Dengue protease is a two-component enzyme and is an important drug target against dengue virus. The protease activity and protein stability of dengue nonstructural protein 3 (NS3) require a co-factor region from a four-span membrane protein NS2B. A natural form of dengue protease containing full-length NS2B and NS3 protease domain NS2BFL-NS3pro will be useful for dengue drug discovery. In current study, detergents that can be used for protease purification were tested. Using a water soluble protease construct, 39 detergents were selected for both NS2B and NS2BFL-NS3pro purification. The results showed that 18 detergents were able to sustain the activity of the natural dengue protease and 11 detergents could be used for NS2B purification. The results obtained in this study will be useful for biochemical and biophysical studies on dengue protease.

1. Introduction

Dengue virus (DENV) together with other *Flaviviridae* family members such as West Nile virus (WNV) is an important human pathogen. DENV affects people all over the world especially in tropical and sub-tropical regions. It is estimated that there are approximately 390 million human infections annually and 96 million cases are with manifest symptoms [1]. DENV infection can cause dengue fever (DF) and some patients can develop serious diseases such as dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) [2].

The genome of DENV is a single-strand, positive-sense RNA and encodes a polyprotein that can be further processed into three structural proteins (capsid, premembrane, and envelope) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) by host and viral proteases [3]. The function of the structural proteins is to form the viral particles. The NS proteins have diverse functions that are necessary for viral replication [3–5]. Host signal peptidase and a viral-encoded protease (NS3) are important for releasing NS proteins by cleaving the junctions between different NS proteins. NS3 is a multi-functional protein containing a N-terminal protease domain (NS3pro) formed by approximately 180 amino acids and C-terminal domain harbouring RNA helicase, and nucleotide triphosphatase (NTP) activities [6–8]. NS3 protease activity requires a co-factor region from NS2B that is a membrane protein containing four transmembrane segments [9]. Due to the importance of NS3 protease activity in viral maturation, dengue protease was a validated drug target [10]. High-throughput drug screening and structure-based drug design have been used to develop NS3 protease inhibitors, but there is no potent inhibitor available [11].

Structural studies revealed that the active pocket of protease is not druggable because of the charges in the active site [11–13]. Developing allosteric inhibitors or inhibitors breaking NS2B and NS3 interaction may be a feasible way to have a potent protease inhibitor [14]. The conventional construct is water soluble and contains a cofactor region from NS2B covalently linked with NS3pro through a Gly_4-Ser-Gly_4 linker (linked protease) [9]. Further studies showed that removal of this artificial linker (un-linked protease) can improve the dynamic nature of the protease [15]. It will be helpful for protease inhibitor development when a natural

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protease is available for biochemical and biophysical studies. As NS2B is a four-span membrane protein [16], a natural form of dengue protease should contain at least full length NS2B (NS2BFL) and NS3pro (NS2BFL-NS3pro). Accumulated studies have demonstrated that this natural dengue protease could be obtained from *Escherichia coli* [17,18]. Although different membrane-mimicking systems such as lipid bilayer, bicelles and nanodisk are efficient systems for membrane protein folding [19–22], a detergent system that does not inhibit protease activity will be helpful for biochemical and biophysical characterization of this natural protease because it can be used to extract protein from the *E. coli* membrane before the protein is reconstituted into different membrane systems or study NS2BFL and NS3pro interaction in vitro. We have shown that lyso-myristoyl phosphatidylcholine (LMPC) can sustain dengue protease activity [17]. We also expressed and purified NS2B into lyso-myristoyl phosphatidylglycerol (LMPG) micelles for structural studies [23]. The protease activity was still low when the natural protease was purified in LMPC or LMPG micelles [17]. Our previous detergent screening was conducted using the following steps. The efficiency of detergents on extraction of dengue protease from *E. coli* membrane was tested followed with measurements of protease activity of purified protease in screened detergents. It is not surprising that screened detergents may not be suitable for enzymatic activity because the screening was based on the efficiency on extracting the target protein from *E. coli* membrane. We then used a modified approach in detergent screening in current study. The effect of detergents on the enzymatic activity of a water soluble protease was first evaluated. Only detergents having no obvious inhibitory effect on protease activity were selected for further protease purification. This method will allow us to identify suitable detergents that can sustain dengue protease activity and can also be used to study the interaction between the NS2BFL and NS3pro in vitro.

In this study, we used a water soluble protease to screen a detergent library containing over 80 detergents to identify candidates that do not inhibit protease activity. There are 39 detergents that showed no inhibitory effect on the water soluble protease. 11 detergents are shown to be suitable for structural and functional studies of NS2B and 18 detergents are suitable for purifying natural dengue protease NS2BFL-NS3pro.

2. Materials and methods

2.1. Expression and purification of un-linked protease

The un-linked protease containing the co-factor region of NS2B and protease domain of NS3 (NS3pro) was expressed and purified as previously described [15]. Briefly, plasmids harbouring NS2B cofactor region and NS3pro were transformed into *E. coli* BL21 (DE3). Protease was induced at 18 °C overnight by adding isopropyl β-D-thiogalactopyranoside (IPTG) to 0.5 mM concentration when the A_{600} reached 0.6. The cells were suspended in a buffer that contained 20 mM Tris–HCl, pH7.8, 300 mM NaCl and 2 mM β-mercaptoethanol. The un-linked protease was purified using a Ni-NTA column and a size exclusion chromatography. The protease was buffer exchanged to a storage buffer that contained 20 mM Tris–HCl, pH7.8, 50 mM NaCl and 1 mM DTT.

2.2. Protease activity using Bz-nKKR-AMC as a substrate

Protease activity assays were performed in 96-well plates. The enzyme activity was measured in an assay buffer that contained 50 mM Tris–HCl, pH 8.0, 20% glycerol in a final volume of 100 μl as previously described [24]. The protease specific, fluorophore-tagged substrate Bz-nKKR-AMC was used in the assay. The substrate concentration in the protease buffer was 60 μM and protease concentration was 20 nM. Detergent was added to the mixture to 1% final concentration. Substrate cleavage was monitored after addition of protease at 37 °C. The increase in fluorescence (excitation 380 nm, emission 450 nm) was continuously monitored on a Tecan Safire 2 microplate reader. The reaction mixture containing no detergent was used as a control.

2.3. Expression of dengue 4 NS2B and the natural protease NS2BFL-NS3pro

The NS2B of DENV 4 was expressed in *E. coli* at 18 °C for 18 h [16]. For the NS2BFL-NS3pro of DENV 4 (Fig. 1), the construct contained full length NS2B and N-terminal protease domain of NS3. The amino acid sequence is shown in Fig. 1. The cDNA of NS2B-NS3pro was synthesized by Genscript and cloned into *Nde*I and *Xho*I sites of pET29b. The resulting plasmid encodes a protein containing NS2BFL, NS3pro fused with a C-terminal tag (LEHHHHHH) for purification. Plasmid was transformed into *E. coli* (BL21DE3) cells. Protein was induced for 18 h by adding IPTG to 0.5 mM final concentration when A_{600} of the cell culture reached 0.8. Cells were harvested by centrifugation at 10,000*g*. Cells were resuspended in a buffer containing 20 mM Tris–HCl, pH7.8, 300 mM NaCl and 2 mM β-mercaptoethanol. Cells were broken in an ice bath by sonication. Sonication was performed using a probe sonicator 4000 package (Misonix) at 5 s on, 5 s off, 40% power to break the cells. The cell debris or inclusion bodies were removed by centrifugation at 3000*g*, 4 °C for 10 min. The resulting supernatant was used for further detergent screening. The NS2B-NS3pro S135A mutation was made by site-directed mutagenesis. The mutant protease was induced using the same protocol as that of the wild type protease.

2.4. Effect of detergents on extracting NS2B and NS2BFL-NS3pro from E. coli membrane

The *E. coli* cell lysate containing NS2B or NS2BFL-NS3pro was mixed with different type of detergents (2%) at 4 °C for 1 h. The mixture was cleared by centrifugation at 20,000*g*, 4 °C for 20 min and the supernatant was loaded in a SDS-PAGE gel for analysis. To test effect of different detergent on protein purification, 1 ml of cell lysate was mixed with different detergent (2% final concentration), followed with centrifugation at 20,000*g*, 4 °C for 20 min. The resulting supernatant was mixed with 50 μl of Ni^{2+}-NTA resin. The resin was washed with a washing buffer that contained 20 mM Tris–HCl, pH7.8, 1% detergent, 20 mM imidazole, and 2 mM β-mercaptoethanol. Protein was eluted with 120 μl of elution buffer containing 500 mM imidazole, pH6.5, 1% detergent and 2 mM β-mercaptoethanol. Samples (6 μl and 4 μl for the purified sample) were separated by SDS-PAGE followed by Western blot using an anti-his antibody (Qiagen) and a second antibody (Genscript). Histidine-tag containing protein was visualized using Bio Rad VersaDoc MP 4000 Molecular Digital Imaging System.

2.5. Purification of NS2BFL-NS3pro using screened detergents

Large-scale protein purification was conducted using a step similar to aforementioned one. *E. coli* cells from 1 L M9 medium were collected and resuspended in a buffer containing 20 mM Tris–HCl, pH7.8, 300 mM NaCl and 2 mM β-mercaptoethanol. Cells were broken by sonication and the cell debris or inclusion bodies were removed by centrifugation at 3000*g*, 4 °C for 10 min. The cell membrane was obtained by ultra-centrifugation at 45,000 rpm in a 70 Ti rotor for 2 h. The pellet was resuspended in a buffer containing 20 mM Tris–HCl, pH7.8, 300 mM NaCl, 2 mM β-