Protein Expression and Purification 133 (2017) 57–65

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Expression, refolding and bio-structural analysis of a tetravalent recombinant dengue envelope domain III protein for serological diagnosis

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ARTICLE INFO

Article history:
Received 28 October 2016
Received in revised form
16 February 2017
Accepted 1 March 2017
Available online 6 March 2017

Keywords:
Dengue
Bacterial expression
Protein refolding
Diagnosis

ABSTRACT

Dengue is a mosquito-borne disease caused by four genetically and serologically related viruses that affect several millions of people. Envelope domain III (EDIII) of the viral envelope protein contains dengue virus (DENV) type-specific and DENV complex-reactive antigenic sites. Here, we describe the expression in *Escherichia coli*, the refolding and bio-structural analysis of envelope domain III of the four dengue serotypes as a tetravalent dengue protein (EDIIIT2), generating an attractive diagnostic candidate. *In vitro* refolding of denatured EDIIIT2 was performed by successive dialysis with decreasing concentrations of chaotropic reagent and in the presence of oxidized glutathione. The efficiency of refolding was demonstrated by protein mobility shifting and fluorescent visualization of labeled cysteine in non-reducing SDS-PAGE. The identity and the fully oxidized state of the protein were verified by mass spectrometry. Analysis of the structure by fluorescence, differential scanning calorimetry and circular dichroism showed a well-formed structural conformation mainly composed of β-strands. A label-free immunoassay based on biolayer interferometry technology was subsequently used to evaluate antigenic properties of folded EDIIIT2 protein using a panel of dengue IgM positive and negative human sera. Our data collectively support the use of an oxidatively refolded EDIIIT2 recombinant chimeric protein as a promising antigen in the serological diagnosis of dengue virus infections.

1. Introduction

Dengue virus (DENV) is a mosquito − borne pathogen causing Dengue Fever (DF) or, in its most severe manifestations, Dengue Hemorrhagic Fever/Dengue Shock Syndrome (DHF/DSS). There are four closely related serotypes of DENV (DENV-1,-2,-3,-4). Infection with one serotype of DENV provides life-long immunity only to that particular serotype. In contrast, secondary infection with another serotype can increase disease severity [24]. The DENV has a positive sense, single stranded RNA genome encoding for a core (C) protein, a membrane (M) protein, an envelope (E) glycoprotein and seven non-structural proteins (NS1 - NS5).

The E protein, a 500 amino acid (aa) residues long polypeptide composed of three structurally distinct domains (EDI, EDII, EDIII), is responsible for a wide range of biological activities, including virions' attachment to host cell receptors and fusion with the endosomal membranes [37]. The E protein is also considered as the primary antigenic site of the DENV and is responsible for inducing neutralizing antibodies and cell mediated immune response in DENV infected hosts [5,6,13,54,57].

From a vaccine and serodiagnosis standpoint, the most important properties of E proteins are associated with EDIII [10,23]. This domain appears to induce strong and specific immune response during infection through neutralizing, type and subtype specific antibodies production [1,9,22,32,38,41,49,52,54]. In a study where a large panel of anti-E monoclonal antibodies was analyzed, it was shown that anti-EDIII antibodies are the most efficient inhibitors of virus infectivity [14]. Furthermore, the EDIII sequence shows a relatively low percentage of sequence identity among Flaviviruses allowing high specificity (limited cross-reaction to non-dengue Flavivirus antibodies) in diagnostic tests [25,26]. EDIII of the 4 serotypes show 70–89% sequence homology [16], and could potentially be used for serological typing of the 4 Dengue strains [17,33].

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http://dx.doi.org/10.1016/j.pep.2017.03.004
1046-5928/

A combination of the four individual Dengue serotype EDIII proteins or a chimeric antigen containing the 4 EDIII sequences have already been shown to be reliable recombinant proteins to detect specific IgM and IgG in the acute and convalescent phases of Dengue disease [3,4,25,40]. However, EDIII contains both linear and conformational epitopes [35] and thus recombinant EDIII should be properly folded to expose structural epitopes and to ensure antibody binding for enhanced sensitivity in a serological assay intended for diagnosis purposes.

Several X-ray crystallography and nuclear magnetic resonance (NMR) studies solved the EDIII structure [16,27,52]. EDIII, which spans amino acids (aa) 296 to 400 of the E protein (DENV1 numbering), is a highly stable, independently folded domain with an immunoglobulin-like structure. Its compact fold is constituted by 9 anti-parallel β-strands linked by random coils and turns [27,36,38,52]. Furthermore, its structural and antigenic integrity depends on a single disulfide bond between the 2 cysteine residues Cys302 and Cys333, well conserved among the 4 Dengue serotypes and other Flaviviruses such as Zika virus, Japanese Encephalitis Virus (JEV) or West Nile Virus (WNV) [10,38,51,58,63]. This internal disulfide linkage is of particular importance for most proteins in the immunoglobulin superfamily and enhances the stability of the Dengue EDIII [64].

Several studies report the production of recombinant single Dengue EDIII proteins using *Escherichia coli (E. coli)* as host but most often, these proteins tend to form insoluble aggregates in the cytoplasm of *E. coli* [19,29,42,48,50,52,61]. Thus, several fusion partners were linked to EDIII to i) enhance protein solubility, ii) facilitate protein purification and iii) enhance biological activity. The fusion partners include, among others, Maltose binding protein (MBP) [21,34,47,53,56,60,46], Glutathione-S-Transferase (GST) [30,55], TrpE [46] or thioredoxin [12]. Partial EDIII solubility was also achieved by adding tags like a N-terminal T7 tag [28] and a N-terminal pelB leader sequence allowing periplasmic protein export [8,64]. Lowering culture temperature and the use of the *E. coli* Origami strain can also help in obtaining small amounts of soluble recombinant EDIII [19,44]. However, only low to moderate yield of soluble recombinant EDIII was achieved with all these strategies and still non-fused proteins are generally preferred for diagnosis purpose as they limit non-specific binding of antibodies. An alternative way to obtain a higher yield of biologically active protein consists in producing proteins in *E. coli* as inclusion bodies and to further apply appropriate refolding methods to improve functional protein recovery [29].

Recent advances in biotechnology describe the design and expression of chimeric recombinant proteins combining several polypeptides of interest in a single artificial polyprotein. This method was used to synthesize EDIII chimeric bivalent and tetravalent constructs containing 2 or 4 EDIII of DENV serotypes respectively for vaccine and diagnostic purposes [7,8,11,18,31,39,40,62]. Although tetravalent chimeric EDIII proteins are well characterized for their antigenic and immunogenic properties [3,4], their structure and thermodynamic properties are, however, poorly described [20].

In the present work, we describe the bacterial production of a recombinant tetravalent chimeric antigen EDIIIT2 intended for accurate Dengue disease diagnosis. We investigated an *in vitro* oxidative refolding process of this multi-epitopic antigen through stepwise dialysis method employing a redox potential that enables disulfide bond formation leading to a fully oxidized state of the structurally important cysteine residues. Intracatenary disulfide bonds formation in EDIIIT2 was verified using fluorescent cysteine labeling and mass spectrometry. Intrinsic tryptophan fluorescence, differential scanning calorimetry and circular dichroism experiments confirmed proper secondary and tertiary structure of this polyprotein constituted of 4 structured domains. The chimeric antigen refolded structure was then correlated with improved antigenic properties. This study demonstrates that, to a large extent, this *in vitro* oxidative refolding process could be a useful method for improved diagnostic performances of bacterially expressed multimeric antigen requiring proper disulfide bond and native-like conformation.

2. Materials and methods

2.1. Bacterial strain, and plasmid

Escherichia coli (*E. coli*) strains BL21 were purchased from Agilent Technologies. Synthetic genes encoding amino acid sequences of the 4 Dengue virus serotypes EDIII were linked with Gly-Ser linker encoding sequences in order to obtain a chimeric gene EDIIIT2. The 4 sequences correspond to the following viral strains: Dengue virus type 1 (strain Nauru/West Pac/1974), Dengue virus type 2 (strain Thailand/16681/1984), Dengue virus type 3 (strain Sri Lanka/1266/2000) and Dengue virus type 4 (strain Dominica/814669/1981). A poly-His tag was inserted at the N-terminal part of the protein to facilitate purification by one step immobilized metal affinity chromatography (IMAC). The optimized synthetic gene for *E. coli* expression of the EDIIIT2 chimeric antigen was bought from a commercial supplier (GeneArt, ThermoFisher Scientific) and ligated into the pMR expression vector [2]. *Bam*HI and *Eco*RI restriction enzymes sites (New England Biolabs) were used to insert the EDIIIT2 gene into the pMR vector. Correct insertion of the chimeric gene into the vector was verified by DNA sequencing.

2.2. Expression and purification

To over-express the protein, the pMR/EDIIIT2 construct was transformed into *E. coli* BL21 competent cells. The recombinant clones were isolated and selected on LB-agar plates (Becton Dickinson) supplemented with 100 μg/mL ampicillin (Merck Millipore #171254). Bacteria carrying the pMR/EDIIIT2 expression vector were grown in 2xYT medium supplemented with 100 μg/mL ampicillin, 1% glycerol and incubated while rotating overnight at 30 °C as a pre-culture. 400 mL of fresh 2xYT medium were then inoculated with a volume of pre-culture to reach an initial 0,2 OD_{600nm} units/mL. Cells were grown at 37 °C until OD_{600nm} reached 0,8 units/mL. Protein expression was induced by the addition of isopropyl-β-D-1-thiogalacto-pyranoside (IPTG) (Roche #11411446001) to a final concentration of 0,5 mM at 30 °C. After 5 h, bacterial cells were harvested by centrifugation at 5000x*g* for 30 min and stored at −80 °C until purification step. Thawed pellet was resuspended in lysis buffer containing 5 units/mL of benzonase (Merck-Novagen #04968117), EDTA-free protease inhibitors (Roche Diagnostics #11873580001) and 2 mM Dithiothreitol (DTT) (Acros Organics). Cells were lysed using a french press at 1,6 kBar and the lysate was centrifuged at 15 000x*g* for 40 min. As EDIIIT2 was principally expressed in inclusion bodies, the centrifuged pellet was re-suspended overnight under magnetic stirring with solubilizing buffer containing 8 M urea and 2 mM DTT. Solubilized inclusion bodies were clarified by centrifugation and 0,8 μm filtration before further purification of the chimeric protein. Clarified supernatant was loaded onto a 5 mL Ni-NTA purification column (Qiagen) under denaturing and reducing conditions. Washing and elution steps were carried out with solubilizing buffer supplemented with 10 mM and 300 mM imidazole (Merck), respectively. Elution fractions containing EDIIIT2 protein were pooled and stored at −80 °C until the refolding step.