CHARACTERIZATION OF FLAVIVIRUS RECOMBINANT PROTEINS

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CHARACTERIZATION OF FLAVIVIRUS RECOMBINANT PROTEINS

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ABBREVIATIONS

APS  ammonium persulfate
Bp   base pair
BCA  bicinchoninic acid
BSA  bovine serum albumin
C    capsid
cDNA complementary DNA
DIII domain III
DENV dengue virus
DNA  deoxyribonucleic acid
dNTPs deoxnucleotide triphosphate
E    envelope
ELISA enzyme-linked immunosorbent assay
ER   endoplasmic reticulum
GACE IgG antibody captured ELISA
FCS  fetal calf serum
HPR  high dengue pooled reference sera
HRP  horseradish peroxidase
IHCM Institute of Health and Community Medicine
IPTG isopropyl thiogalactosidase
JEV  Japanese encephalitis virus
Kb   kilobase
kDa  kilodalton
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<th>Term</th>
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<td>KUNV</td>
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<td>LB</td>
<td>Luria Bertani</td>
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<tr>
<td>M</td>
<td>membrane</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>MVEV</td>
<td>Murray Valley encephalitis virus</td>
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<tr>
<td>NS</td>
<td>non-structural</td>
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<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>prM</td>
<td>premembrane</td>
</tr>
<tr>
<td>PNR</td>
<td>pooled negative reference sera</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RO</td>
<td>reverse osmosis</td>
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<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<tr>
<td>TEMED</td>
<td>N,N,N',N' - tetramethylethylenediamine</td>
</tr>
<tr>
<td>TPB</td>
<td>tryptose phosphate broth</td>
</tr>
<tr>
<td>UHQ</td>
<td>ultra high quality</td>
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<td>WNV</td>
<td>West Nile virus</td>
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ABSTRACT

The envelope protein of the flavivirus consists of three distinct domains named domain I, II, and III. Secondary structure of envelope protein shows that this protein is folded in such a way that makes the domain III protein separate from domains I and II. This has made domain III protein possible to be cloned on its own because of continuity in gene sequence compared to domains I and II which overlaps with each other. Besides that, function of domain III as a receptor binding domain and its ability to elicit neutralizing antibodies when challenged has made this protein interesting to study. Here, we cloned and expressed the domain III of eight different flaviviruses which are of DENV1-4, WNV, JEV, KUNV and MVEV. The domain III fragment was cloned into pET-SUMO cloning vector and the expression was done in a bacterial expression system. All the domain III proteins were expressed as a fusion protein to histidine-tag protein. The expression of domain III was confirmed by probing a western blot with Ni-HRP which detects the presence of his-tag in the recombinant protein. The reactivity test done on these recombinant domain III proteins had shown that they were reactive when probed with high positive pooled dengue reference sera (HPR). These domain III proteins were then purified using nickel affinity chromatography before characterization work were performed. The purified products were tested in indirect IgG ELISA and the results were compared to the GACE which uses native antigens. The result shows that the sensitivity of domain III based assay is only 55.28%, however the specificity is 91.70%. The domain III proteins were also tested in latex beads agglutination assay, however results were worse in terms of sensitivity and specificity. The results obtained suggest that domain III is not a good candidate for use in diagnostic assay in place of authentic antigen. However, due to the simpler work involve in constructing the
recombinant protein, its still can be used for other various functional studies. Interestingly, neutralization test using domain III positive pooled serum shows nearly 10 fold higher neutralizing antibodies titer compared to domain III negative pooled, indicating that this domain might be a good candidate in developing an antiviral agent or vaccine for preventing the infections by flavivirus.
keputusan yang diperolehi menunjukkan bahawa protei domain III tidak begitu sesuai untuk penggunaan dalam asai diagnostik. Walau bagaimanapun, disebabkan protei rekombinan ini lebih mudah untuk disediakan, maka ia masih boleh digunakan untuk kajian-kajian berfungsi yang lain. Menariknya, ujian peneutralan menggunakan sampel positif domain III memberikan hampir 10 kali ganda titer antibodi peneutralan dibandingkan dengan sampel negatif domain III. Ini menunjukkan bahawa, domain ini mungkin boleh digunakan untuk menghasilkan ejen antivirus ataupun vaksin bagi menghalang jangkitan oleh virus flavi.
CHAPTER 1: INTRODUCTION

1.1: Flavivirus

Flaviviruses are small single-stranded positive-sense RNA viruses that are transmitted primarily by arthropods, usually mosquitoes and ticks. The genus, once classified in the family Togaviridae, now constitutes one of three genera in the family Flaviviridae; the other two genera are Pestivirus and Hepacivirus. Viruses within the genus are categorized into clades, clusters, and species, according to molecular phylogenetics or into antigenic complexes and subcomplexes based on classic serological criteria (Calisher et al., 1989). Phylogenetic analyses of the 72 species of flaviviruses have shown 14 clades, which in turn can be grouped into three clusters: the mosquito-borne cluster, the tick-borne cluster and the no-vector cluster. However, all flaviviruses of human importance belong to the first two clusters (Kuno et al., 1998).

Flaviviruses can cause a variety of syndromes ranging from benign febrile illness to severe systemic diseases with hemorrhagic fever or major organ involvement. Entities of major global concern include dengue virus (DENV) with its associated dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), Japanese encephalitis virus (JEV), West Nile virus (WNV), and Yellow Fever virus (YFV). Other flaviviruses of regional or endemic concern include Murray Valley encephalitis virus (MVEV), St. Louis encephalitis virus (SLEV), and tick-borne encephalitis virus (TBEV). Decreases in mosquito control efforts during the latter part of the 20th century, coupled with societal factors (e.g., increased transportation and
dense urbanization) have contributed to the re-emergence of flaviviruses such as DENV in South and Central America (Lindenbach et al., 2007). More recently, other flaviviruses have emerged in new geographic regions and caused epidemics of human and/or animal disease, for example the introduction and subsequent spread of West Nile virus (WNV) in North America (Kilpatrick et al., 2006).

1.2: Genome Structure

The genome of flaviviruses is a single-stranded molecule, positive sense RNA of about 11 kb in size. This molecule contains a short untranslated region at 3' and 5' ends, which are known as the non-polyadenylated 3' terminus and the 5' cap (Wengler and Wengler, 1981). Flavivirus proteins are produced by translation of a single, long open reading frame (ORF) to generate a polyprotein. There are also a complex series of post-translational proteolytic cleavages of the polyprotein which are achieved by a combination of host and viral proteases, to generate mature viral proteins. The viral polyprotein is cleaved to generate three structural proteins (capsid, C; membrane protein, prM/M; and envelope, E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Sumiyoshi et al., 1987). The structural proteins are presented in the N-terminal region of the polyprotein, while the non-structural proteins are located in the C-terminal region of the polyprotein. Figure 1.2.1 shows the details of flavivirus genome structure.
Figure 1.2.1: Flavivirus genome structure and expression. A: Genome structure and RNA elements. The viral genome is depicted with the structural and nonstructural protein coding regions, the 5' cap, and the 5' and 3' noncoding regions (NCR) indicated. B: Polyprotein processing and cleavage products. Boxes below the genome indicate precursors and mature proteins generated by the proteolytic processing cascade. C: The proposed topology of the flavivirus polyprotein cleavage products with respect to the endoplasmic reticulum (ER) membrane is shown (reproduced from Lindenbach et. al., 2007).
1.3: Viral Protein

1.3.1: Capsid protein

The molecular mass of capsid (C) protein is approximately 11kDa. It is a highly basic or positively charged protein. Charged residues are clustered at the N- and C-termini, separated by an internal hydrophobic region that mediates membrane association (Ma et al., 2004). The capsid protein also contains a C-terminal hydrophobic anchor that functions as a signal peptide for endoplasmic reticulum (ER) translocation of prM. This hydrophobic region is cleaved from mature C by the viral serine protease (Lobigs, 1993).

1.3.2: Membrane protein

There are two forms of membrane protein, the precursor of M protein (prM) and membrane protein (M). The prM protein is a glycoprotein of about 26kDa. It is found in the intracellular immature virions and translocated into the ER by the C-terminal hydrophobic domain of capsid protein. A major function of prM protein is to prevent envelope (E) protein from undergoing acid-catalyzed rearrangement during transit through the secretory pathway. M protein is a much smaller protein of about 7-8 kDa in size. It is located in extracellular mature virions. The conversion of immature virus particles to mature virions occurs in the secretory pathway, together with cleavage of prM into pr- and M fragments. The cleavage of prM results in the
rearrangement of the virion surface, which makes the mature virions infectious (Wengler and Wengler, 1989).

1.3.3: Envelope protein

Envelope (E) protein is the major protein on the surface of flavivirus virions. The size of E protein is about 53kDa and it is synthesized as a type 1 membrane protein containing 12 conserved cysteines that form disulfide bonds (Nowak and Wengler, 1987)). Secretion of E protein together with proper folding and stabilization in low pH, is depends on the coexpression with the prM protein (Konishi and Mason, 1992). This protein is glycosylated in all flaviviruses except in Kunjin and West Nile virus. The native form of E folds into an elongated structure which is rich in β-sheets. This structure will form a head to tail homodimers that lie parallel with the virus envelope (Rey et. al., 1995). Each subunit of E protein consists of three distinct domains (I, II, III). Domain I, which forms a β-barrel, contains type-specific nonneutralizing epitopes and is believed to be the hinge region engaged in low-pH induced structural changes. Domain II, an elongated dimerization domain, involves in cell membrane fusion following virus entry and contains many cross-reactive epitopes eliciting neutralizing and nonneutralizing monoclonal antibodies. Domain III which maintains an immunoglobulin-like fold, involves in receptor binding and contains multiple type and subtype-specific epitopes eliciting virus neutralizing antibodies (Rey et. al., 1995; Crill and Roehrig, 2001).