



รายงานวิจัยฉบับสมบูรณ์

โครงการ THE DENGUE VIRUS PROTEASE COMPLEX NS2B/NS3 -- A
NOVEL TARGET FOR THE STRUCTURE -- BASED DESIGN
OF IMPROVED ANTIVIRAL AGENTS

โดย Asst. Prof. Dr. Gerd Katzenmeier

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ผู้วิจัย

Asst. Prof. Dr. Gerd Katzenmeier

สังกัด

สถาบันอณูชีววิทยาและพันธุศาสตร์
มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

FINAL PROJECT REPORT

**THE DENGUE VIRUS PROTEASE COMPLEX NS2B/NS3 – A NOVEL
TARGET FOR THE STRUCTURE – BASED DESIGN OF IMPROVED
ANTIVIRAL AGENTS**

PRINCIPAL INVESTIGATOR: GERD KATZENMEIER

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Gerd Katzermeier

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site gave no detectable cleavage product, which is consistent with a strict requirement of the NS3 protease for a basic residue at this position.

In order to exclude the possibility that co-purification of a bacterial protease with similar specificity was responsible for the proteolysis we have observed with the dibasic peptide substrates, an enzymatically inactive mutant of NS3 was generated by introducing a S135A substitution in the NS3 sequence. The inactive mutant protein was expressed at levels comparable to the wild-type enzyme and pure NS2B-NS3(pro)M protein was prepared under equivalent conditions. Control reactions containing NS2B-NS3(pro)M showed absolutely no proteolytic activity after incubation up to 8 hours with all substrates thereby confirming that the preparations of NS2B-NS3(pro) were free of contaminating bacterial proteases.

Activity of the enzyme was dependent on the salt concentration and 50% decrease of activity was observed in the presence of 0.1 M NaCl. The enzyme exhibited remarkable thermostability. Incubation prior to the enzymatic assay up to 8 hrs. at room temperature (25°C) did not affect activity significantly.

Cloning and expression of NS3 in yeast. An alternative expression system for the DEN protease was generated in the methylotrophic yeast *Pichia pastoris* [27, 28]. The NS3(pro) gene was amplified by PCR using a *E. coli* expression vector encoding the NS3 protein as a template. The gene segment was cloned into the yeast expression plasmid pPICZ α A generating a N-terminal fusion with the yeast α -factor and a C-terminal fusion with the polyHis affinity tag. Restriction analysis and DNA sequencing confirmed the correct sequence of the recombinant plasmid. *Pichia pastoris* strain KM71H was transformed by electroporation and genomic integration was confirmed by PCR. After transformation of *Pichia pastoris* KM71H with pPICZ α A/NS3(pro), the target protein was detected immunochemically in cell lysates after column purification, but not in the medium supernatant obtained from fermentation under MeOH induction. In the presence of 6 M urea, most of the contaminating proteins were efficiently removed. A 45 kDa protein present in SDS-PAGE gels corresponds to NS3(pro) fused to the yeast α -factor and is indicative of incomplete and inefficient cleavage at the KEX2 site. However, a weak signal observed at 22 kDa could represent a small amount of protein with proteolytically removed α -factor. Secretion of this protein into the medium was not observed. Multiple bands which are cross-reactive in the Western blot also indicated the presence of glycosylated forms of α -NS3(pro). Incubation of the expressed protein with endoglycosidase H gave a product band with a 10 kDa smaller apparent molecular weight. Immuno-positive bands between 22 and 45 kDa could consist

of various products of proteolytic degradation. The results show, that the dengue NS3 protease domain can be efficiently expressed in *Pichia*, however, a secretion system facilitating protein purification remains to be established.

Within the framework of this project, progress has been made towards establishing a research infrastructure for the biochemical and molecular biological characterization of the dengue virus NS3 protease and tools have been generated and methods have been introduced into our group which allow a more detailed analysis of structure - activity relationships for this important viral drug target. Moreover, the project has identified novel objectives and research strategies which can be implemented in the future to further enhance our understanding of the mechanistic role of the NS2B/NS3 protease in flavivirus replication and pathogenesis.

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OUTPUT

Publications:

1. Champreda, V., Khumthong, R., Subsin, B., Angsuthanasombat, C., Panyim, S. and Katzenmeier, G. (2000) The two-component protease NS2B-NS3 of dengue virus type 2: cloning, expression in *Escherichia coli* and purification of the NS2B, NS3(pro) and NS2B-NS3 proteins. *J. Biochem. Mol. Biol.* **33**, 294-299.
2. Khumthong, R., Angsuthanasombat, C., Panyim, S. and Katzenmeier, G. (2001). Enzymatic *in vitro* determination of the dengue virus NS2B-NS3 protease activity with fluorescent peptide substrates. (*submitted*)
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STUDENTS GRADUATED IN M.Sc. WITHIN THE PROJECT:

<u>NAME</u>	<u>DATE OF GRADUATION</u>	<u>THESIS TITLE</u>
Benchamas Subsin	October 5, 1998	Molecular cloning, expression in E. coli and partial purification of the dengue virus type 2 protease complex NS2B/NS3.
Rabuesak Khumthong	May 26, 1999	Expression and purification of the protease complex NS2B/NS3 from the dengue virus type 2
Veerawat Champreda	May 28, 1999	Molecular cloning, expression in E. coli and purification of the serine protease domain of NS3 protein and the NS2B protein of dengue virus serotype 2 strain 16681.
Rungrutai Udomsinprasert	October 22, 1999	Molecular cloning, expression in E. coli and purification of a native dengue type 2 cleavage site probe for the detection of NS3 protease activity.
Rooge Suvannasuthi	May 31, 2001	Expression, purification and biochemical characterization of the dengue virus type 2 serine protease domain NS3(pro).

The Two-Component Protease NS2B-NS3 of Dengue Virus Type 2: Cloning, Expression in *Escherichia coli* and Purification of the NS2B, NS3(pro) and NS2B-NS3 Proteins

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without a bound cofactor resembles the structure of the HCV protease in its co-complex with the NS4A cofactor. The dengue virus NS2B-NS3 protease complex is considered to be a primary target for the design of antiviral agents, which inhibit polyprotein processing, and hence are equally effective against all 4 dengue virus serotypes (Patick and Potts, 1998). The dengue virus polyprotein processing has been studied extensively with virus-derived vectors in transiently infected cell lines and by immunoprecipitation of polyproteins. However, these methods are not suitable for the real-time detection of enzymatic activity in inhibitor screening assays. As a prerequisite for the development of *in vitro* assay systems for the dengue virus NS3 protease, we describe here a method for the overexpression in *E. coli* and biochemical purification of the components of the dengue virus protease complex NS2B and NS3 by using a polyhistidine affinity tag and a metal chelate column chromatography. Three recombinant proteins derived from the DEN 2 polyprotein were constructed: i) the 14 kDa NS2B cofactor, ii) a truncated form of NS3 containing 184 amino acid residues of the N-terminal protease domain (NS3pro) and iii) the 84 kDa full-length NS2B-NS3 fusion protein. The availability of these DEN 2 recombinant proteins will assist the optimization of assay reaction conditions for the NS3 protease.

Material and Methods

Reagents and general methods. All recombinant DNA and cloning procedures were carried out by standard methods (Sambrook *et al.*, 1989). Restriction endonucleases and DNA modifying enzymes were obtained from Gibco BRL (Gaithersburg, USA), Stratagene (La Jolla, USA), and New England Biolabs (Beverly, USA), and used according to the manufacturer's recommendations. Cloned *Pfu* DNA polymerase was from Stratagene and enterokinase from Boehringer (Mannheim, Germany). Ni²⁺-iminodiacetic acid (IDA) resin (Probond™) was from Invitrogen (San Diego, USA) and Ni²⁺-NTA alkaline phosphatase conjugate was obtained from Qiagen (Chatsworth, USA).

Plasmid expression constructs. All dengue virus polyprotein sequences were generated by PCR. Oligonucleotide primers used for PCR were obtained from Bio-Synthesis (Lewisville, USA) and PCR reactions were run on a thermal cycler Gene Amp System 2400 (Perkin Elmer, Norwalk, USA). Two half-genome cDNA clones of DEN 2 strain 16681 in plasmid pBluescript II KS

constructed by RT PCR (Nopporn Sitthisombut, unpublished data) were used for the amplification of NS2B and NS3(pro) sequences. Plasmid pD2/IC-30P (Kinney *et al.*, 1997) was obtained from Dr. Sirithorn Butrapet and used as a template for the amplification of NS2B-NS3. Primer sequences and binding sites on the dengue virus genome are shown in Table 1. PCR products were analysed by restriction enzyme digestion and all PCR amplified regions were verified by DNA sequencing on an ABI Model 377 DNA Sequencer (Perkin Elmer). DEN 2 sequences were subcloned into expression vector pTrcHisA (NS2B-NS3) to give pTH/NS2B-3 and pTrcHisB to give pTH2B, and pTH3p, respectively. Expression constructs were transformed into *E. coli* host strains TOP10 (Invitrogen), JM109, C41(DE3) (Miroux and Walker, 1996) and BL21(DE3)pLysS.

Expression, purification and on-column refolding of dengue virus 2 proteins. DEN 2 fusion proteins were purified in analogy to published procedures (Crowe *et al.*, 1996; Shi *et al.*, 1997). *E. coli* cells, transformed with expression plasmids, were grown in a LB medium (500 ml) containing ampicillin (100 µg/ml) at 37°C until the optical density at 600 nm reached 0.6. The expression was induced with isopropyl-β-D-thiogalacto-pyranoside (IPTG, 0.1 mM) for 6-8 h. Cells were harvested by centrifugation, resuspended in 15 ml phosphate buffered saline buffer containing lysozyme (100 µg/ml) and lysed in a French pressure cell at 14,000 p.s.i. The lysate was treated with RNaseA and DNase at 5 µg/ml and was kept on ice for 15 min. Inclusion bodies were harvested by centrifugation and the pellet fraction was washed 3 times with 20 mM sodium phosphate, pH 7.4, 1% (v/v) triton X-100. Inclusion bodies were solubilized either in 15 ml guanidinium lysis buffer (6 M guanidinium hydrochloride, 20 mM sodium phosphate, pH 7.8, 500 mM NaCl) or in 15 ml buffer A (8 M urea, 20 mM sodium phosphate, pH 7.8, 500 mM NaCl). The solution was clarified by centrifugation at 4°C and applied to a Probond Ni²⁺-affinity column (Invitrogen) containing 2 ml of resin preequilibrated with 20 ml of buffer A. Column elutions were performed by gravitational flow at a rate of approximately 0.5 ml/min. The column was washed twice with 5 ml of buffer A, followed by a washing with 20 ml of buffer B (buffer A with 20 mM imidazole). The column was washed with 5 ml of buffer C (buffer A without urea) and was kept overnight at 4°C. Contaminates weakly bound to the resin were removed by 2 subsequent washes with buffer C containing 50 mM and 100 mM imidazole. DEN proteins fused to the polyHis tag were eluted from the column by using a step gradient of 10 ml of buffer C containing 500 mM imidazole. Fractions of 1 ml were collected

Table 1. PCR amplified regions of the DEN 2 genome and sequences of PCR primers. DEN 2 map positions refer to Kinney *et al.*, 1997. F and R indicate forward and reversed primer sequences, respectively. Restriction sites introduced for cloning are underlined. Stop codons engineered in the sequences are shown in small letters

DEN 2 Region	Genome Position	PCR Primer Sequence
NS2B	4132-4521	F: 5'-CAAGAACCAGCAAGGATCCGAGCTGGCC-3' R: 5'-CCCACAAGGATCCcctaCCGTTGTTTCTTCA-3'
NS3(pro)	4522-5073	F: 5'-GAAGTGAAGAAGGATCCCGCCGGAGTATTGTG-3' R: 5'-CATGATGGTCGGGATCCTcctaTCGGAAAATGTC-3'
NS2B-NS3	4132-6366	F: 5'-GAACCAGCAAGGATCCAGCTGGCCATTAAATGAGGC-3' R: 5'-GTGATTAGGTTTCGAGCTCAGcctaCTTCTTCCGGCTGC-3'

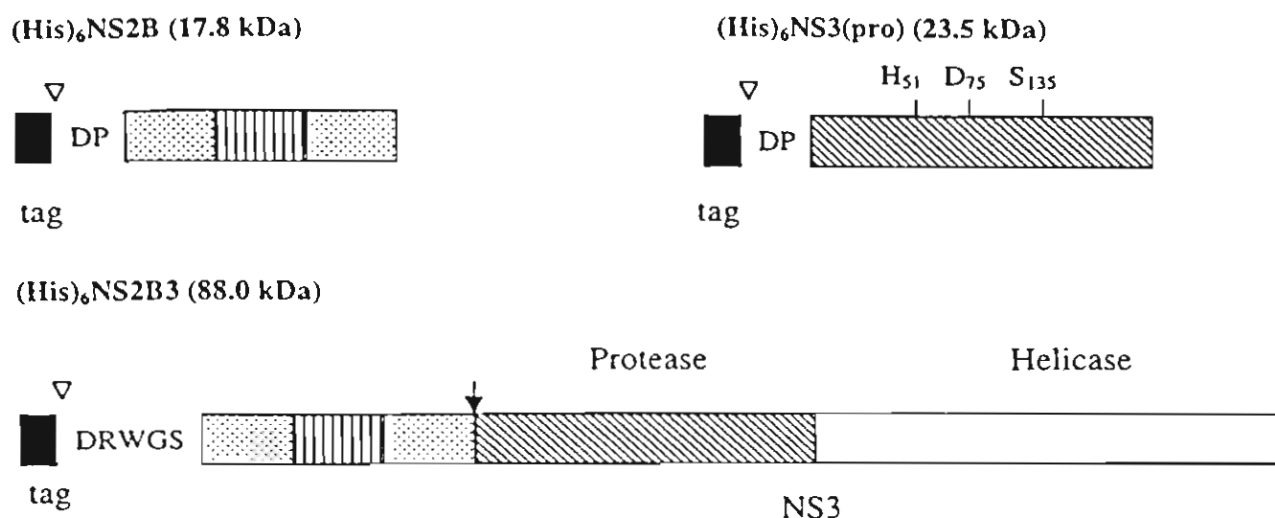


Fig. 1. Structures of the the DEN 2 expression constructs in pTrcHis plasmid vectors. Shown are 3 recombinant molecules; NS2B, NS3(pro) and NS2B-3. The polyHis affinity tag is shown as a black box, the DEN 2 NS3 protease cleavage site is indicated by a bold arrow, the enterokinase cleavage sites are shown by an open triangle. Regions with different enzymatic activities in NS3 are indicated. Hydrophobic regions in NS2B are represented as dotted boxes and the hydrophilic activation domain is depicted by vertical bars. NS3(pro) is shown as a hatched box and the position of the catalytic triad residues is indicated. Additional amino acid residues, inserted between the polyHis tag and the DEN proteins, are shown.

and the elution profile was monitored at A_{280} . Peak fractions were pooled and desalted by using a PD10 column (Pharmacia) equilibrated with 50 mM Tris-HCl, pH 7.4. Protein concentrations were determined using a Bio-Rad protein quantitation kit and samples were analyzed on SDS-PAGE. Proteins separated on gels were probed for the presence of the polyHis fusion tag on Western blots with Ni^{2+} -NTA conjugated to alkaline phosphatase. Preparations were stored in 50 mM Tris-HCl, pH 7.4, 40% glycerol at -20°C .

Results and Discussion

Sequences encoding dengue virus type 2 nonstructural proteins NS2B, NS3(pro) and NS2B-NS3 were amplified by PCR using DEN 2 genomic cDNA plasmids as a template and initially cloned into pUC18. The PCR amplified regions were analyzed by DNA sequencing and the sequence of NS2B revealed one silent mutation (genome position 4344: T \rightarrow C), while sequences of NS3(pro) and NS2B-NS3 were identical to the sequences reported for dengue type 2 strain 16681 (Kinney *et al.*, 1997). Dengue sequences were subcloned into the expression vector pTrcHis, where they were fused in frame to a 3.5 kDa affinity tag containing a stretch of 6 consecutive histidine residues (His_6). Physical maps of the resulting expression constructs are shown in Fig. 1.

Several *E. coli* host strains, including TOP10, JM109, C41(DE3) and BL21(DE3)pLysS, were transformed with expression plasmids. With all host strains, expression of the DEN 2 virus fusion proteins was observed after 4 to 6 h of IPTG induction and polypeptides of the expected sizes were produced predominantly as inclusion bodies. However, the amounts of DEN 2 fusion proteins produced were dependent on the host strain and relatively higher in the *ompT* protease

deficient strains BL21 and C41(DE3) when compared to JM109 and TOP10. *E. coli* C41(DE3) is a mutant derivative of *E. coli* BL21(DE3), which has been shown to allow the accumulation of high amounts ($> 1 \text{ mg/l}$ bacterial culture) of hydrophobic membrane proteins (Miroux and Walker, 1996). We also observed 2- to 4-fold reduced growth rates with the expression products in the host strain *E. coli* BL21 that was grown under IPTG induction when compared to the host strain grown in the absence of IPTG (data not shown). Membrane permeabilization effects that lead to decreased growth have been reported for *E. coli* BL21 overexpressing nonstructural proteins of the Japanese encephalitis virus. This virus is a closely related flavivirus that contains the NS2B-NS3 protease complex (Chang *et al.*, 1999). It is conceivable that similar effects on membrane integrity are exerted by the hydrophobic sequences contained in the overexpressed DEN 2 proteins. This may explain the relatively low yields we obtained, in particular with the NS2B protein.

An easy-to-perform and uniform purification protocol was developed for the three recombinant proteins from DEN 2. Inclusions were fractionated into a soluble supernatant and an insoluble pellet fraction. Inclusion bodies were detergent-extracted and after solubilization with 6 M guanidinium hydrochloride or 8 M urea, the solubilized material was loaded on a Ni^{2+} -metal chelate affinity column. Contaminated proteins were removed by washing with buffers containing 50 and 100 mM imidazole, respectively. Since the target proteins were produced as an inclusion body, a refolding step was necessary. DEN 2 proteins were renatured on-column by incubating the resin in a binding buffer without urea at 4°C . Proteins were then eluted with 500 mM imidazole. A representative purification is shown in Fig. 2 for the NS3(pro)

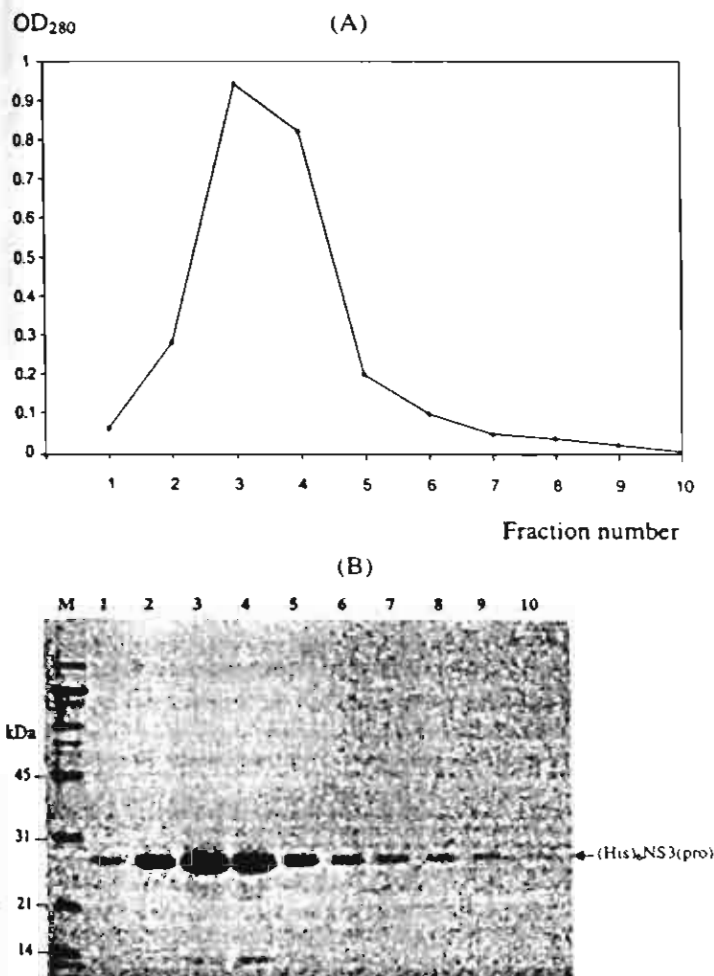


Fig. 2. Metal chelate affinity chromatography of NS3(pro) on a Ni²⁺-IDA column. See details in Materials and Methods. (A) Inclusion bodies solubilized in the presence of 8 M urea were applied to a ProBond column. For on-column refolding of the protein, urea was removed by washing with a binding buffer without urea and the column was kept overnight at 4°C. The His₆ fusion protein was eluted with a step gradient of 10 ml buffer containing 500 mM NaCl, 20 mM sodium phosphate pH 7.8, 500 mM imidazole. Fractions of 1 ml were collected and the elution profile was monitored at A₂₈₀. (B) SDS-PAGE analysis of elution fractions from the metal affinity column. Samples (15 µl) were mixed with 5 µl of the SDS gel sample buffer (0.2 M Tris-HCl, pH 7.5, 10% SDS, 0.025% bromophenol blue, 2.5% glycerol, 100 mM DTT) for PAGE on 12.5% polyacrylamide gels using a Bio-Rad Mini-Protein II cell. Numbers above the lanes correspond to column fraction numbers. M indicates the position of the M_r marker proteins in kDa.

protein. Elution profiles were similar for all the proteins under investigation. This indicates that separation was largely based on the presence of the polyHis tag. The purification method gave comparable results when working in a batch-mode rather than with a column-bound resin. Imidazole and NaCl were removed by using a PD10 desalting column and protein samples were subjected to SDS-PAGE. Figure 3 shows

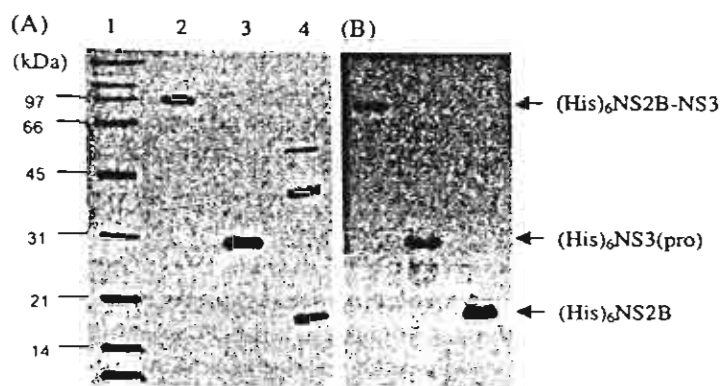


Fig. 3. Purification of DEN 2 recombinant proteins from *E. coli*. (A) PAGE and Coomassie staining of a 12.5% gel. Lane 1: molecular weight marker; lane 2: (His)₆NS2B-3 (88 kDa), lane 3: (His)₆NS3(pro) (23.5 kDa) and lane 4: (His)₆NS2B (17.8 kDa) proteins purified from *E. coli* strain BL21 by metal affinity chromatography. (B) Corresponding Western blot analysis of DEN 2 fusion proteins purified by ProBondTM chromatography. Fusion proteins were probed with Ni-NTA conjugated to alkaline phosphatase at 1 : 1000 dilution.

the Coomassie-stained SDS-PAGE analysis of the protein purification for NS2B, NS3(pro) and NS2B-NS3. The proteins were obtained with yields of 0.5, 6.0, and 1.0 mg/l *E. coli* culture for NS2B, NS3(pro) and NS2B-NS3, respectively. All DEN proteins appear in Coomassie-stained gels as single bands at the expected molecular size. The proteins were recovered at a level of purity of nominally 90% as judged by the densitometry of Coomassie blue-stained gels (using a Bio-Rad Gel Doc 1000 gel imaging system) and protein concentrations in peak fractions. In the corresponding Western blot for NS2B, NS3(pro) and NS2B-NS3 probed with Ni²⁺-NTA conjugated to alkaline phosphatase, strong signals were observed with polypeptides of the expected molecular size. However, (His)₆NS2B was associated with minor amounts of impurities at molecular weights of approximately 40 and 60 kDa, which did not crossreact in the Western blot analysis. (His)₆NS2B-NS3 was obtained as a single band at 88 kDa. This indicates that this protein was purified as a single chain fusion protein and was not autoprocessed at the NS2B/NS3 cleavage site or cleaved at the internal site ...RR ▽ GR... (residues 457 to 460 in the DEN 2 sequence) within the helicase portion of NS3 (Teo and Wright, 1997).

The polyhistidine tag could not be cleaved off the purified DEN 2 fusion proteins by using enterokinase in the presence of 20% (v/v) glycerol, 1% (v/v) triton X-100, or urea and NaCl at concentrations that are compatible with enterokinase activity. Inefficient removal of the affinity tag has been observed with a number of flavivirus nonstructural proteins overexpressed in *E. coli* (Khromykh *et al.*, 1996). However, because of the small size, the hexahistidine tag rarely affects the function of the tagged protein. DEN 2 NS3 polypeptides, expressed with a C-terminal fusion of a (His)₆ tag, have been shown to possess nucleoside triphosphatase and RNA helicase

activities *in vitro* (Li et al., 1999).

The protease complex NS2B-NS3 is essential for the maturation of infectious virus and thus represents a target for the design of selective antiviral therapeutics. For hepatitis C virus NS3 protease, a number of assays based on synthetic peptide substrates have been developed which are potentially applicable for high-throughput screening of novel protease inhibitors (Cerretani et al., 1999; Liu et al., 1999; Zhang et al., 1999). Currently, the enzymatic activity of the recombinant dengue virus protease components is under investigation in our laboratory by using peptide substrates with native dengue virus cleavage site sequences and the results will be published elsewhere.

The method presented here allows the purification to near-homogeneity of the dengue virus nonstructural proteins NS2B, NS3(pro) and NS2B-NS3 with a single-step metal affinity chromatography separation. This will facilitate the preparation of quantitative amounts of the components of the dengue virus NS2B-NS3 protease complex. These are required for assay development, characterization of the biochemical reaction parameters of the NS3 protease and the identification of useful inhibitors for this important biomedical target.

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***IN VITRO* DETERMINATION OF DENGUE VIRUS TYPE 2 NS2B–NS3 PROTEASE ACTIVITY WITH FLUORESCENT PEPTIDE SUBSTRATES**

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Abstract

Dengue virus, a member of the *Flaviviridae* family, generates individual viral proteins by processing of a polyprotein precursor catalysed by host cell and virus proteases. Enzymatic activity of the purified NS2B-NS3(pro) protease complex was determined *in vitro* with dansyl-labeled peptide substrates based upon native dengue virus type 2 cleavage sites. Substrate peptides and the cleavage products could be separated by reversed-phase HPLC and were identified by UV and fluorescence detection. All 12mer peptide substrates representing the DEN polyprotein junction sequences at the 2A/B, 2B/3, 3/4A and 4B/5 sites were cleaved by the recombinant protease NS2B-NS3(pro). Enzymatic activity was dependent on the salt concentration and the enzyme was relatively thermostable. Relative rates for the hydrolysis of the peptide substrates indicate the order 4B/5 > 3/4A > 2A/2B >> 2B/3 for the cleavage efficiency at individual polyprotein sites. Our results show that NS3 protease activity of the refolded NS2B-NS3(pro) protein can be assayed *in vitro* with high sensitivity by using cleavage-junction derived peptide substrates.

Keywords: Dengue virus, NS3 protein, serine protease, peptide, HPLC, assay.

INTRODUCTION

Dengue virus (DEN), a member of the *Flaviviridae* family, is the etiologic agent of dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Rigau-Perez *et al.*, 1998). The global pandemic of dengue virus has intensified over the past 15 years and infections with dengue virus affect now estimatedly 100 million people in more than 100 tropical and subtropical countries (Monath, 1994; Gubler and Clark, 1995). At present, there is no antiviral therapy available for the prevention and treatment of acute dengue virus infections. Although monovalent and tetravalent vaccines are currently being evaluated (Huang *et al.*, 2000; Bhamarapavati and Sutee, 2000), the development of novel inhibitors against viral target enzymes for the causative treatment of dengue diseases is urgently required.

Dengue virus type 2 (DEN 2), one of 4 serotypes, contains a 10.7 kb single stranded RNA genome of positive strand polarity with a type I cap at the 5' terminus. The RNA genome is translated into a large polyprotein precursor of 3,391 amino acid residues in DEN 2 with proteins arranged in the order NH₂ - C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 - COOH (Chambers *et al.*, 1990). Proteolytic processing in the region of the structural proteins is mediated by a host cell signal peptidase located within the endoplasmic reticulum (Markoff, 1989; Nowak *et al.* 1989) and cleavages at the NS2A - 2B, NS2B - 3, NS3 - 4A and NS4B - 5 sites are catalyzed by the virus - encoded two-component protease NS2B-NS3 (Ryan *et al.*, 1998). Cleavage at the NS1/NS2A and NS4A/B site is mediated by a host cell - derived protease (Falgout and Markoff, 1995). In addition to the cleavages catalysed at the polyprotein junctions, the viral protease mediates cleavages within the viral proteins C, NS4A and within NS3 itself (Arias *et al.*, 1993; Lin *et al.*, 1993; Lobigs, 1993; Teo and Wright, 1997).

Based on sequence comparisons between DEN NS3 and a number of eukaryotic and viral serine proteases, a protease domain (*flavivirin*, EC 3.4.21.91) encoded within the N-terminal part of the 70 kDa NS3 protein and a catalytic triad comprised of residues His-51, Asp-75 and Ser-135 in DEN 2 has been identified (Bazan and Fletterick, 1989). The C-terminal two-thirds of the NS3 protein have been shown to contain a polynucleotide-stimulated NTPase and RNA helicase enzymatic activity (Kadare and Haenni, 1997). The functional domains of the serine protease and the RNA-stimulated NTPase activity overlap within a region of 20 amino acid residues and the minimum domain size for the protease has been mapped by a mutational analysis to 167 residues of NS3 (Li *et al.*, 1999). The NS2B-NS3 two-component protease catalyses cleavages within the viral polyprotein at 'dibasic' sites containing Lys-Arg, Arg-Arg, Arg-Lys and - at the NS2B/NS3 site - Gln-Arg followed by a small side chain residue, Gly, Ala and Ser, at the P1' position.