Journal of Biochemistry and Molecular Biology, Vol. 33, No. 4, July 2000, pp. 294-299

JOURNAL OF
Biochemistry
and
Molecular Biology

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

Veerawat Champreda, Rabuesak Khumthong, Benchamas Subsin, Chanan Angsuthanasombat, Sakol Panyim and Gerd Katzenmeier*

Institute of Molecular Biology and Genetics, Mahidol University, Salaya Campus, Phutthamonthon No. 4 Rd., Nakornpathom 73170, Thailand

Received 24 March 2000, Accepted 8 May 2000

Proteolytic processing of the dengue virus serotype 2 polyprotein precursor is catalyzed by a host signal peptidase and a virus encoded two-component protease consisting of the nonstructural proteins, NS2B and NS3. We expressed in *Escherichia coli* the NS2B, NS3(pro) and NS2B-NS3 proteins from the dengue virus type 2 strain 16681 as N-terminal fusions with a hexahistidine affinity tag under the control of the inducible *trc* promoter. All fusion proteins were purified to >90% purity by detergent extraction of inclusion bodies and a single step metal chelate chromatography. Proteins were refolded on-column and recovered with yields of 0.5, 6.0 and 1.0 mg/l of *E. coli* culture that was grown to OD_{600} = 1.0 for NS2B, NS3(pro) and NS2B-NS3, respectively. Purified proteins gave strong signals in Western blots using Ni^{2+}-nitrilotriacetic acid as a probe for the presence of the polyHis tag. During the purification process, $(His)_6$NS2B-NS3 was apparently not autoproteolytically cleaved at the NS2B/NS3 site.

Keywords: Dengue virus type 2, Metal chelate affinity chromatography, NS2B, NS3, Purification, Serine protease.

Introduction

Dengue virus generates mature viral proteins by co- and posttranslational proteolytic processing of a polyprotein precursor catalyzed by host cell and virus proteases (Chambers *et al.*, 1990a; Ryan *et al.*, 1998). Dengue virus type 2 (DEN 2), a member of the *Flaviviridae* family, has a single stranded RNA genome of 10,723 nucleotides that encodes a single polyprotein precursor of 3,391 amino acid residues. The polyprotein is processed into 3 structural proteins (C, prM, E) and at least 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). The NS2B-NS3 protease complex is responsible for cleavages *in cis* at the NS2A/NS2B and NS2B/NS3 sites and for cleavages *in trans* at the NS3/NS4A and NS4B/NS5 sites (Chambers *et al.*, 1990b; Preugschat *et al.*, 1991). Reports have been made of the cleavages catalyzed by the NS3 protease within the viral proteins C, NS4A as well as within a conserved C-terminal region of NS3 itself (Arias *et al.*, 1993; Lin *et al.*, 1993; Lobigs, 1993; Teo and Wright, 1997; Shoji *et al.*, 1999). The 70 kDa NS3 protein contains two domains that are associated with multiple enzymatic activities. A serine protease is located in the N-terminal 180 amino acid residues (Bazan and Fletterick, 1989; Chambers *et al.*, 1990b; Preugschat *et al.*, 1990), whereas the RNA helicase and RNA-stimulated nucleoside triphosphatase are associated with the C-terminal region of the protein (Gorbalenya *et al.*, 1989; Wengler and Wengler, 1993; Kadare and Haenni, 1997). The protease and NTPase enzymatic functions of the NS3 protein share a region of overlapping activities between residues 160 and 180 (Li *et al.*, 1999). The 14 kDa amphipathic NS2B protein acts as an activator of the protease by the formation of a heterodimeric complex with NS3 (Falgout *et al.*, 1991). A central hydrophilic domain of 40 amino acid residues has been identified as an activation region for the protease (Falgout *et al.*, 1993). Based on the modelling data, a putative association sequence comprised of the residues 70-81 of NS2B has been proposed for NS3 binding (Brinkworth *et al.*, 1999). Crystal structures for the DEN 2 protease domain and for the NS3 protease of hepatitis C virus (HCV), an intensively characterized member of the *Flaviviridae* that is complexed with the virus-encoded cofactor NS4A, have been resolved (Kim *et al.*, 1996; Yan *et al.*, 1998; Krishna Murthy *et al.*, 1999). Both proteases display the two six-stranded β-barrel domains characteristic of serine proteases of the chymotrypsin family. Also, the structure of the DEN 2 protease

*To whom correspondence should be addressed.
Tel: +66-2-441-9003; Fax: +66-2-441-9906
E-mail: FRKGZ@mahidol.ac.th

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). Ni^{2+}-iminodiacetic acid (IDA) resin (Probond™) was from Invitrogen (San Diego, USA) and Ni^{2+}-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 Ni^{2+}-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'-CCCACAAGGATCCctaCCGTTGTTTCTTCA-3'
NS3(pro)	4522-5073	F: 5'-GAAGTGAAGAAGGATCCCGCCGGAGTATTGTG-3'
		R: 5'-CATGATGGTCGGGATCCTctaTCGGAAAATGTC-3'
NS2B-NS3	4132-6366	F: 5'-GAACCAGCAAGGATCCAGCTGGCCATTAAATGAGGC-3'
		R: 5'-GTGATTAGGTTCGAGCTCAGctaCTTTCTTCCGGCTGC-3'

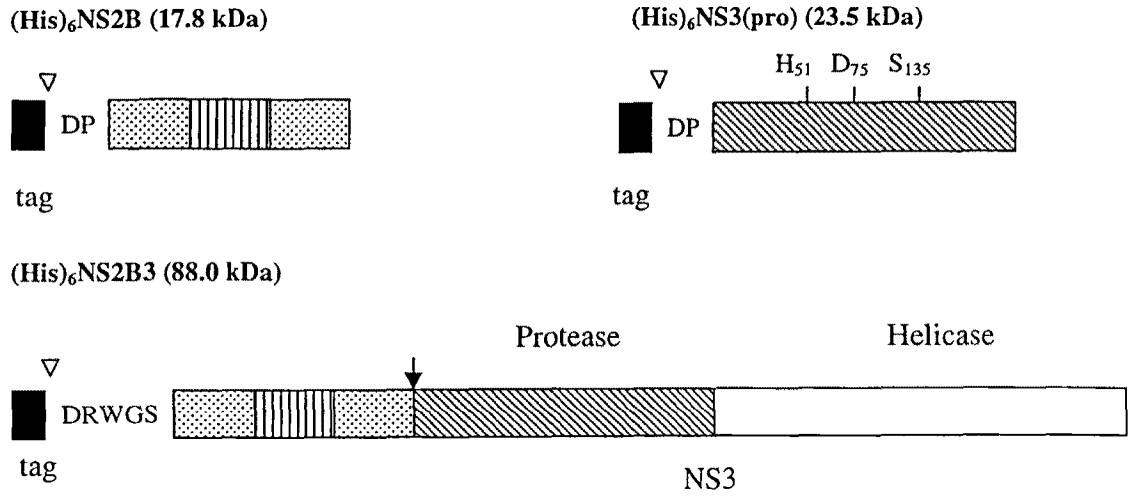


Fig. 1. Structures of the the DEN 2 expression constructs in pTrcHis plasmid vectors. Shown are 3 recombinant molecules; NS2B, NS3(pro) and NS2B-3. The polyHis affinity tag is shown as a black box, the DEN 2 NS3 protease cleavage site is indicated by a bold arrow, the enterokinase cleavage sites are 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.

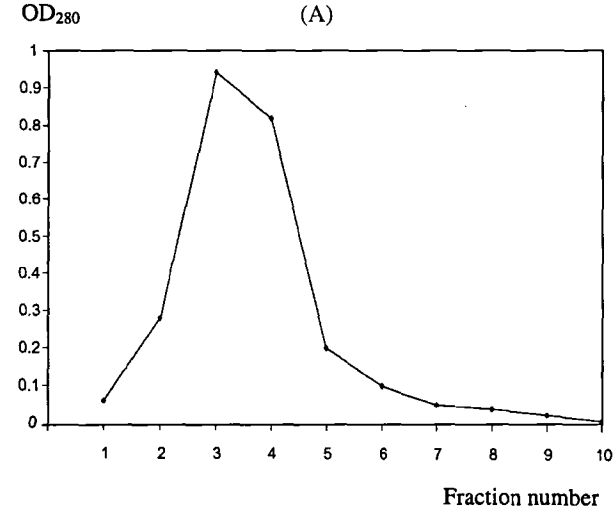
and the elution profile was monitored at A_{280}. Peak fractions were pooled and desalted by using a PD10 column (Pharmacia) equilibrated with 50 mM Tris-HCl, pH 7.4. Protein concentrations were determined using a Bio-Rad protein quantitation kit and samples were analyzed on SDS-PAGE. Proteins separated 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 → 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 *omp*T 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 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)



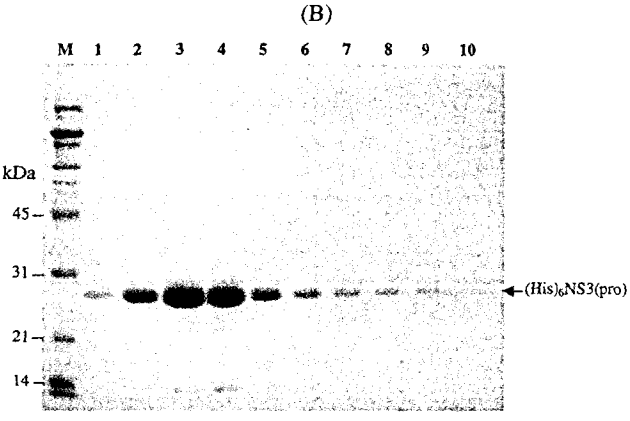


Fig. 2. Metal chelate affinity chromatography of NS3(pro) on a Ni^{2+}-IDA column. See details in Materials and Methods. (A) Inclusion bodies solubilized in the presence of 8 M urea were applied to a Probond column. For on-column refolding of the protein, urea was removed by washing with a binding buffer without urea and the column was kept overnight at 4°C. The His_6 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 A_{280}. (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-Protean 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

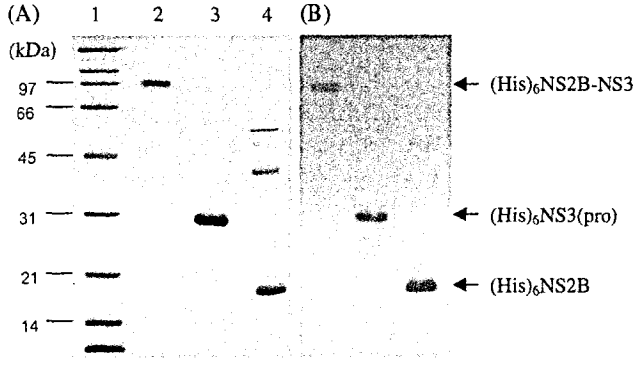


Fig. 3. Purification of DEN 2 recombinant proteins from *E. coli*. (A) PAGE and Coomassie staining of a 12.5% gel. Lane 1: molecular weight marker; lane 2: $(His)_6$NS2B-3 (88 kDa), lane 3: $(His)_6$NS3(pro) (23.5 kDa) and lane 4: $(His)_6$NS2B (17.8 kDa) proteins purified from *E. coli* strain BL21 by metal affinity chromatography. (B) Corresponding Western blot analysis of DEN 2 fusion proteins purified by ProBond™ 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 Ni^{2+}-NTA conjugated to alkaline phosphatase, strong signals were observed with polypeptides of the expected molecular size. However, $(His)_6$NS2B 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. $(His)_6$NS2B-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.

Acknowledgments We thank Drs. Nopporn Sitthisombut (Dept. of Microbiology, Chiang Mai University, Thailand) for the dengue virus type 2 cDNA clones, Sirithorn Butrapet (Center for Vaccine Development, Mahidol University, Nakornpathom, Thailand) for plasmid pD2/IC-30P and John E. Walker (The Medical Research Council Laboratory of Molecular Biology, Cambridge, UK) for a gift of *E. coli* C41(DE3). We thank Dr. Albert J. Ketterman for his helpful discussions. This work was supported by grants from the National Science and Technology Development Agency (NSTDA) and the Thailand Research Fund (TRF).

References

Arias, C. F., Preugschat, F. and Strauss, J. H. (1993) Dengue 2 virus NS2B and NS3 form a stable complex that can cleave NS3 within the helicase domain. *Virology* **193**, 888-899.

Bazan, J. F. and Fletterick, R. J. (1989) Detection of a trypsin-like serine protease domain in flaviviruses and pestiviruses. *Virology* **171**, 637-639.

Brinkworth, R. I., Fairlie, D. P., Leung, D. and Young, P. R. (1999) Homology model of the dengue 2 virus NS3 protease: putative interactions with both substrate and NS2B cofactor. *J. Gen. Virol.* **80**, 1167-1177.

Cerretani, M., Di Renzo, L., Serafini, S., Vitelli, A., Gennari, N., Bianchi, E., Pessi, A., Urbani, A., Colloca, S., De Francesco, R., Steinkuehler, C. and Altamura, S. (1999) A high-throughput radiometric assay for hepatitis C virus NS3 protease. *Anal. Biochem.* **266**, 192-197.

Chambers, T. J., Hahn, C. S., Galler, R. and Rice C. M. (1990a) Flavivirus genome organization, expression and replication. *Annu. Rev. Microbiol.* **44**, 649-688.

Chambers, T. J., Weir, R. C., Grakoui, A., McCourt, D. W., Bazan, J. F., Fletterick R. J. and Rice, C. M. (1990b) Evidence that the N-terminal domain of nonstructural protein NS3 from yellow fever virus is a serine protease responsible for site-specific cleavages in the viral polyprotein. *Proc. Natl. Acad. Sci. USA* **87**, 8898-8902.

Chang, Y.-S., Liao, C.-L., Tsao, C.-H., Chen, M.-C., Liu, C.-I., Chen, L.-K. and Lin, Y.-L. (1999) Membrane permeabilization by small hydrophobic nonstructural proteins of Japanese encephalitis virus. *J. Virol.* **73**, 6257-6264.

Crowe, J., Masone, B. S. and Ribbe, J. (1996) One-step purification of recombinant proteins with the 6x His tag Ni-NTA resin; in *Methods in Molecular Biology*, Harwood, A. (ed.), pp. 491-510, Humana Press, Totowa, New Jersey.

Falgout, B., Pethel, M., Zhang, Y. M. and Lai, C.-J. (1991) Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. *J. Virol.* **65**, 2467-2475.

Falgout, B., Miller, R. H. and Lai, C.-J. (1993) Deletion analysis of dengue virus type 4 nonstructural protein NS2B: identification of a domain required for NS2B-NS3 protease activity. *J. Virol.* **67**, 2034-2042.

Gorbalenya, A. E., Koonin, E. V., Donchenko, A. P. and Blinov, V. M. (1989) Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. *Nucleic Acids Res.* **17**, 4713-4730.

Kadare, G. and Haenni, A. L. (1997) Virus-encoded RNA helicases. *J. Virol.* **71**, 2583-2590.

Khromykh, A. A., Harvey, T. J., Abedinia, M. and Westaway, E. G. (1996) Expression and purification of the seven nonstructural proteins of the flavivirus Kunjin in the *E. coli* and the baculovirus expression systems. *J. Virol. Methods* **61**, 47-58.

Kim, J. L., Morgenstern, K. A., Lin, C., Fox, T., Dwyer, M. D., Landro, J.A., Chambers, S. P., Markland, W., Lepre, C. A., OMalley, E. T., Harbeson, S. L., Rice, C. M., Murcko, M. A., Caron, P. R. and Thomson, J. A. (1996) Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. *Cell* **87**, 343-355.

Kinney, R. M., Butrapet, S., Chang, G. J., Tsuchiya, K. R., Roehrig, J. T., Bhamarapravati, N. and Gubler, D. J. (1997) Construction of infectious cDNA clones for dengue 2 virus strain 16681 and its attenuated vaccine derivative, strain PDK-53. *Virology* **230**, 300-308.

Krishna Murthy, H. M., Clum, S. and Padmanabhan, R. (1999) Dengue virus NS3 serine protease. *J. Biol. Chem.* **274**, 5573-5580.

Li, H., Clum, S., You, S., Ebner, K. E. and Padmanabhan, R. (1999) The serine protease and RNA-stimulated nucleoside triphoshatase and RNA helicase functional domains of dengue virus type 2 NS3 converge within a region of 20 amino acids. *J. Virol.* **73**, 3108-3116.

Lin, C., Amberg, S. M., Chambers, T. J. and Rice, C. M. (1993) Cleavage at a novel site in the NS4A region by the yellow fever virus NS2B-3 proteinase is a prerequisite for processing at the downstream 4A/4B signalase site. *J. Virol.* **67**, 2327-2335.

Liu, Y., Kati, W., Chen, C. M., Tripathi, R., Molla, A. and Kohlbrenner, W. (1999) Use of a fluorescence plate reader for measuring kinetic parameters with inner filter effect correction. *Anal. Biochem.* **267**, 331-335.

Lobigs, M. (1993) Flavivirus premembrane protein cleavage and spike heterodimer secretion require the function of the viral proteinase NS3. *Proc. Natl. Acad. Sci. USA* **90**, 6218-6222.

Miroux, B. and Walker, J. E. (1996) Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J. Mol. Biol.* **260**, 289-298.

Patick, A. K. and Potts, K. E. (1998) Protease inhibitors as antiviral agents. *Clin.* Microbiol. Rev. **11**, 614-627.

Preugschat, F., Lenches, E. M. and Strauss, J. H. (1991) Flavivirus enzyme-substrate interactions studied with chimeric proteinases: identification of an intragenic locus important for substrate recognition. *J. Virol.* **65**, 4749-4758.

Preugschat, F., Yao, C. W. and Strauss, J. H. (1990) *In vitro* processing of dengue virus type 2 nonstructural proteins NS2A, NS2B, and NS3. *J. Virol.* **64**, 4364-4374.

Ryan, M. D., Monaghan, S. and Flint, M. (1998) Virus-encoded proteinases of the Flaviviridae. *J. Gen. Virol.* **79**, 947-959.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd ea., Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.

Shi, P. J., Maizels, N. and Weiner, A. M. (1997) Recovery of soluble, active recombinant proteins from inclusion bodies. *BioTechniques* **23**, 1036-1038.

Shoji, I., Suzuki, T., Sato, M., Aizaki, H., Chiba, T., Matsuura, Y. and Miyamura, T. (1999) Internal processing of hepatitis C virus NS3 protein. *Virology* **254**, 315-323.

Teo, K. F. and Wright, P. J. (1997) Internal proteolysis of the NS3 protein specified by dengue virus type 2. *J. Virol.* **72**, 624-632.

Wengler, G. and Wengler, G. (1993) The NS3 nonstructural protein of flaviviruses contains an RNA triphosphatase activity. *Virology* **197**, 265-273.

Yan, Y., Li, Y., Munshi, S., Sardana, V., Cole, J. L., Sardana, M., Steinkuehler, C., Tomei, L., De Francesco, R., Kuo, L. C. and Chen, Z. (1998) Complex of NS3 protease and NS4a peptide of BK strain hepatitis C virus: a 2.2 A resolution structure in a hexagonal crystal form. *Protein Sci.* **7**, 837-847.

Zhang, R., Beyer, B. M., Durkin, J., Ingram, R., Njoroge, F. G., Windsor, W. T. and Malcolm, B. A. (1999) A continuous spectrophotometric assay for the hepatitis C virus serine protease. *Anal. Biochem.* **270**, 268-275.