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In Vitro Determination of Dengue Virus Type 2 NS2B-NS3 Protease Activity with Fluorescent Peptide Substrates

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The NS2B-NS3(pro) polyprotein segment from the dengue virus serotype 2 strain 16681 was purified from overexpressing *E. coli* by metal chelate affinity chromatography and gel filtration. Enzymatic activity of the refolded NS2B-NS3(pro) protease complex was determined *in vitro* with dansyl-labeled peptide substrates, based upon native dengue virus type 2 cleavage sites. The 12mer substrate peptides and the cleavage products could be separated by reversed-phase HPLC, and were identified by UV and fluorescence detection. All of the peptide substrates (representing the DEN polyprotein junction sequences at the NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5 sites) were cleaved by the recombinant protease NS2B-NS3(pro). No cleavage was observed with an enzymatically inactive S135A mutant of the NS3 protein, or with a modified substrate peptide of the NS3/NS4A polyprotein site that contained a K2093A substitution. Enzymatic activity was dependent on the salt concentration. A 50% decrease of activity was observed in the presence of 0.1 M sodium chloride. Our results show that the NS3 protease activity of the refolded NS2B-NS3(pro) protein can be assayed *in vitro* with high specificity 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 last 15 years, and infections with the dengue virus are now estimated to affect 100 million people in more than 100 tropical and subtropical countries (Monath, 1994; Gubler and Clark, 1995). At present, there is no antiviral therapy that is available for the prevention and treatment of acute dengue virus infections. Although monovalent and tetravalent vaccines are currently being evaluated (Bhamarapravati and Sutee, 2000; Huang *et al*., 2000), the development of novel inhibitors against viral target enzymes for the causative treatment of dengue diseases is a major focus of dengue research.

The 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 that are arranged in the order NH_2-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 that is located within the endoplasmic reticulum (Markoff, 1989; Nowak *et al*. 1989). Cleavage at the NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 sites are catalyzed by the virus-encoded two-component protease NS2B-NS3 (*for review see* Ryan *et al*., 1998). Cleavage at the NS1/NS2A and NS4A/NS4B sites are mediated by a host cell-derived protease (Falgout and Markoff, 1995). In addition to the cleavages that are catalyzed at the polyprotein junctions, the viral protease mediates the cleavage 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) that is encoded within the N-terminal part of the 70 kDa NS3 protein, and a catalytic triad that is 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 has been

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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; the minimum domain size for the protease has been mapped by mutational analysis to 167 residues of NS3 (Li *et al*., 1999). The NS2B-NS3 two-component protease catalyses the cleavage within the viral polyprotein at 'dibasic' sites. These contain 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.

The protease forms a heterodimeric complex with the small 14 kDa protein NS2B (Falgout *et al*., 1991), which acts as a cofactor for NS3 activation. A region that is critical for the activation of NS3 has been mapped to a 40 amino acid residue charged central domain within NS2B of DEN 4 (Falgout *et al*., 1993). A central region within this hydrophilic domain shows some sequence homology to the NS4A activating peptide of the HCV NS3 protease. Molecular modeling has provided evidence that this subdomain (encompassing the sequence GSSPILISITE) could be sufficient for the activation of the NS3 protease (Brinkworth *et al*., 1999).

The crystal structure of the dengue virus NS3 protease domain has been resolved at a 2.1 A resolution (Murthy *et al*., 1999). The overall conformation of the DEN 2 NS3 N-terminal domain without the NS2B cofactor resembles the structure of the hepatitis C virus (HCV) NS3-NS4A co-complex (Kim *et al*., 1996). However, a structural zinc binding site, and a hydrophobic stretch of amino acids at the N-terminus, are not present in the DEN 2 structure.

Recently, an *in vitro* assay by using a cloned polyprotein segment and fluorescent tripeptides as substrates for the NS3 protease was described (Yusof *et al*., 2000). The NS2B(H)-NS3(pro) protein, consisting of the hydrophilic core fragment of NS2B fused to the NS3 protease domain, exhibited autoproteolytic cleavage at the NS2B/NS3 cleavage site after purification and refolding from *E. coli*. The presence of the NS2B core sequence was shown to be indispensable for cleavage of a native NS4B/NS5 polyprotein substrate. The presence of NS2B(H) resulted in a several thousand-fold activation of the NS3 protease towards dibasic peptide substrates. In contrast, the cleavage of the chromogenic protease model substrate, N-α-benzoyl-L-arginine-p-nitroanilide (BAPA), was catalyzed by NS3(pro) with higher specific activity when compared to the NS2B(H)-NS3 co-complex. These findings provide evidence to suggest the existence of two discrete conformations of the enzyme with different specificities within the NS3(pro) protease and the heterodimeric NS2B-NS3 complex.

We reported earlier the cloning and biochemical purification of the dengue virus type 2 two-component protease (Champreda *et al*., 2000). Here we describe a HPLC-based enzymatic *in vitro* assay for the NS2B-NS3(pro) protein that is fused to a N-terminal polyHis affinity tag by using fluorescence-labeled peptides representing native dengue polyprotein cleavage sequences. The assay allows the simultaneous analysis of the substrate and product peptides that are produced by the enzymatic reaction with high sensitivity and specificity.

Materials and Methods

Plasmids and site-directed mutagenesis The DNA sequence that encodes the dengue virus type 2 strain 16681 polyprotein segment (genome position 4132-5073) was obtained by PCR using the dengue virus cDNA plasmid pD2/IC-30P (Kinney *et al*., 1997) as a template. The DNA segment was cloned into the expression vector pTrcHisA (Invitrogen, San Diego, USA) and transformed into the *E. coli* host strain C41(DE3), as described previously (Champreda *et al*., 2000). The enzymatically inactive mutant NS2B-NS3(pro)M with a S135A exchange in the NS3 sequence was generated by site-directed mutagenesis following the procedure of the QuickChange™ mutagenesis kit (Stratagene, La Jolla, USA). Complementary mutagenic oligonucleotide primers 5'-CCTGGAA CTGCAGGATCTCCAATTATCG-3' and 5'-CGATAATTGGAGA TCCTGCAGTTCCAGG-3' simultaneously introduced a *Pst* I site that was suitable for restriction screening (purchased from Genset Inc., Singapore). PCR reactions were run on a thermal cycler Gene Amp System 2400 (Perkin Elmer, Norwalk, USA). The sequence of the mutated NS2B-NS3(pro) gene was verified by DNA sequencing using an ABI prism 377 sequencer (Perkin Elmer).

Purification of DEN 2 NS2B-NS3(pro) and NS2B-NS3(pro)M proteins The recombinant NS2B-NS3(pro) and NS2B-NS3(pro)M proteins of the dengue virus type 2 were expressed as inclusion bodies in bacterial cells *E. coli* C41. They were purified by a modification of the method described previously (Champreda *et al.*, 2000). The pellet fraction was washed 3 times with cold 50 mM Tris-Cl, pH 7.8, 1% (v/v) Triton X-100 and solubilized by resuspension in 15 ml of 100 mM Tris-Cl, pH 7.8, 500 mM NaCl, 6 M guanidinium hydrochloride. The denatured lysate was clarified by centrifugation at 4°C. The proteins were fractionated by passage through a HiTrap chelating Ni^{2+}-affinity column (Pharmacia, Uppsala, Sweden) that contained 1 ml of resin that was equilibrated with binding buffer A (20 mM Tris-Cl, pH 7.8, 500 mM NaCl, 6 M guanidinium hydrochloride). The column was washed with 10 ml of a binding buffer, and was subsequently washed with 20 ml of buffer B (buffer A with 50 mM imidazole). The proteins were eluted from the column at a flow rate of 0.5 ml min^{-1} in buffer C (100 mM Tris-Cl, pH 7.8, 500 mM NaCl, 6 M guanidinium hydrochloride, 500 mM imidazole). The fractions of 1 ml were collected and the elution profile was monitored at A_{280}. Fractions were analyzed by electrophoresis on 15% polyacrylamide gels. Peak fractions were pooled and applied to a Superdex 200 HR10/30 gel filtration column (Pharmacia). The column was eluted at a flow rate of 0.2 ml min^{-1} with 24 ml of an elution buffer (100 mM Tris-Cl, pH 8.3, 300 mM NaCl, 6 M guanidinium hydrochloride). Fractions of 0.5 ml were collected and analyzed by SDS-PAGE. Elution fractions that contained the DEN proteins were pooled and the proteins were refolded by stepwise dialysis against 4 changes of a 200 ml dialysis buffer (100 mM Tris-HCl, pH 8.0, 300 mM NaCl)

for 20 h at 4°C in order to remove guanidinium hydrochloride. The presence of the NS2B-NS3(pro) and NS2B-NS3(pro)M proteins was detected in Coomassie-blue stained SDS-PAGE gels, and by Western blotting on nitrocellulose filters using an anti-polyhistidine affinity tag antiserum (Invitrogen). The band intensities of the proteins were analyzed by densitometry using a Bio-Rad Geld Doc 1000 gel imaging system. The protein concentrations in the samples were determined by using a Bio-Rad protein quantitation kit with BSA as a standard. The purified protease preparations were stored in 150 mM NaCl, 40% glycerol, 50 mM Tris-HCl, pH 8.0 at –20°C.

Peptide substrates The 12mer peptide substrates (encompassing the native dengue virus type 2 strain 16681 polyprotein NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 cleavage sites containing a N-terminal dansyl group (Dns)) were commercially synthesized (Biosynthesis Inc., Lewisville, USA) and purified by reversed-phase HPLC. A 6mer cleavage product peptide Dns-FAAGRK of the NS3/NS4A site was used as a retention reference for HPLC analysis. The monobasic peptide Dns-FAAGRASLTLNL that contained a lysine to alanine substitution at the P1 position served as a negative control in the cleavage assays with DEN NS3 protease. The peptides were solubilized in water or DMSO.

HPLC DEN NS3 protease assays Reaction mixtures for the detection of NS3 proteolytic activity were composed of 100 µM Tris-HCl, pH 8.0, that contained 10 mM of peptide substrate and 2-4 µg of purified NS2B-NS3(pro) protein in a final volume of 40 µl. Cleavage controls contained trypsin (Sigma Chemistry, St. Louis, USA) at a concentration of 5 µg/ml. The samples were incubated at room temperature (25°C) for various periods of time, and the reactions were quenched by the addition of acetonitrile to give a final concentration of 20%. The samples were loaded on a reversed-phase HPLC (Waters) and separated on a C-18 phenomenex column (250 mm × 4.6 mm, Pharmacia) that was equilibrated with 0.1% (v/v) TFA/water. The reaction products were separated by a linear gradient of 2% min^{-1} of 0.1% TFA/acetonitrile. The products were analyzed by UV-detection at 214 and 280 nm and by a fluorescence monitor (Waters 474) set to the excitation wavelength λ = 340 nm and the emission wavelength λ = 510 nm. The extent of hydrolysis was estimated from the decrease in the area of the substrate peak. An injection of standard amounts of the substrate was used in order to calibrate peak areas.

Results and Discussion

A number of reports in literature describe HPLC-based discontinuous assays for the analysis of the enzymatic activity of viral proteases by using synthetic peptide substrates (Kakiuchi *et al*., 1997; Seybert *et al*. 1997; Wang *et al*., 1997). In analogy to these procedures, we used a series of synthetic peptides that represent the authentic proteolytic cleavage sites of the dengue virus serotype 2 as substrates to analyze the enzymatic activity of a purified NS2B-NS3(pro) protease *in vitro*.

The dengue virus NS2B-NS3(pro) that fused to a N-terminal 6xHis purification tag was purified from overexpressing *E. coli* C41 harboring the pTrcHis/2B-3(pro) expression plasmid (Fig. 1). In contrast to the purification of the DEN protease components, which we described earlier (Champreda *et al.*, 2000), the NS2B-NS3(pro) polyprotein precursor that was obtained from a single-step metal chelate column chromatography was associated with substantial amounts of impurities at a molecular weight between 30 and 45 kDa. These could not be removed efficiently with Ni-affinity chromatography. Therefore, it was necessary to include a second fractionation step by using a Superdex 200 gel filtration column in order to obtain NS2B-NS3(pro) with sufficient purity for the enzymatic assay. The purified protein, which was recovered from chromatography under denaturing conditions, was subsequently refolded by stepwise dialysis. Fig. 2 shows a SDS-PAGE gel of a representative purification of the NS2B-NS3(pro) protein. A single immunoreactive band at 45 kDa molecular weight was observed in Western blots by using anti-polyhistidine antibodies as a probe for the presence of the 6xHis tag (data not shown). By a comparison of the band intensities on Coomassie blue-stained gels, the amount of NS2B-NS3(pro) that was produced per liter of the *E. coli* culture that was grown to OD_{600} = 1.0 was estimated to be 1 mg. NS2B-NS3(pro) was recovered at a purity of >95%, as estimated from a densitometry analysis of SDS-PAGE band intensities.

In order to demonstrate enzymatic activity of the NS2B-NS3(pro) fusion protein, 12-amino-acid peptides with sequences that correspond to the NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 cleavage sites of the DEN 2 polyprotein were chemically synthesized and N-terminally labeled with a dansyl group to facilitate photometric detection by UV and fluorescence detection. Purified NS2B-NS3(pro)

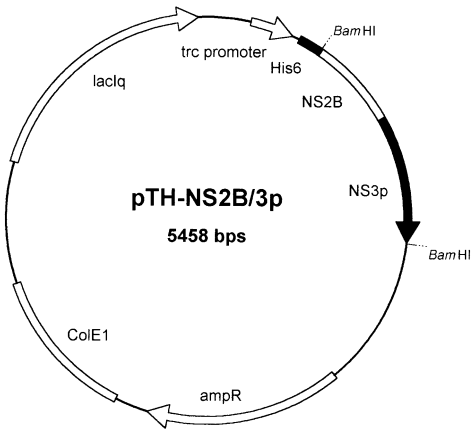

Fig. 1. Overexpression of the DEN NS2B-NS3(pro) protease in *E. coli*. The DNA-segment that encodes the NS2B-NS3(pro) gene of the dengue virus type 2 was generated by PCR using the DEN cDNA plasmid pD2/IC-30P (Kinney *et al*., 1997) as a template. The segment was cloned into the expression vector pTrcHis, and fused to a N-terminal 6xHis affinity purification tag. The resulting construct is under the control of the trc-promoter, and expression is induced by IPTG.

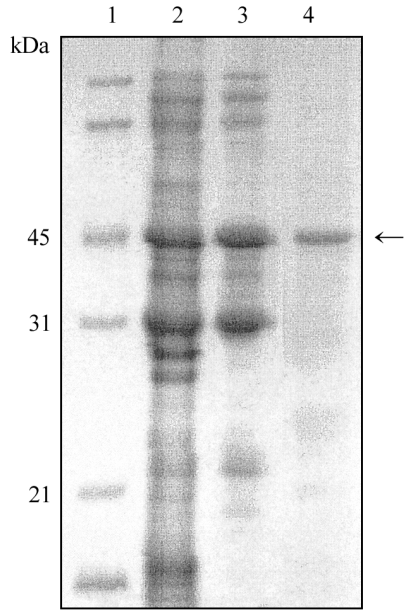

Fig. 2. Coomassie-stained 12.5% SDS-PAGE gel analysis of the DEN NS2B-NS3(pro) protease purification. Lane 1: molecular weight marker (kDa); lane 2: solubilized inclusion bodies from *E. coli*; lane 3: elution fraction from a HiTrap metal chelate affinity column that was obtained in the presence of 0.5 M imidazole; lane 4: peak fraction from chromatography on Superdex 200 HR10/30 after extensive dialysis against 100 mM Tris-Cl, pH 8.0, 300 mM NaCl. The arrow indicates the position of the NS2B-NS3(pro) protein.

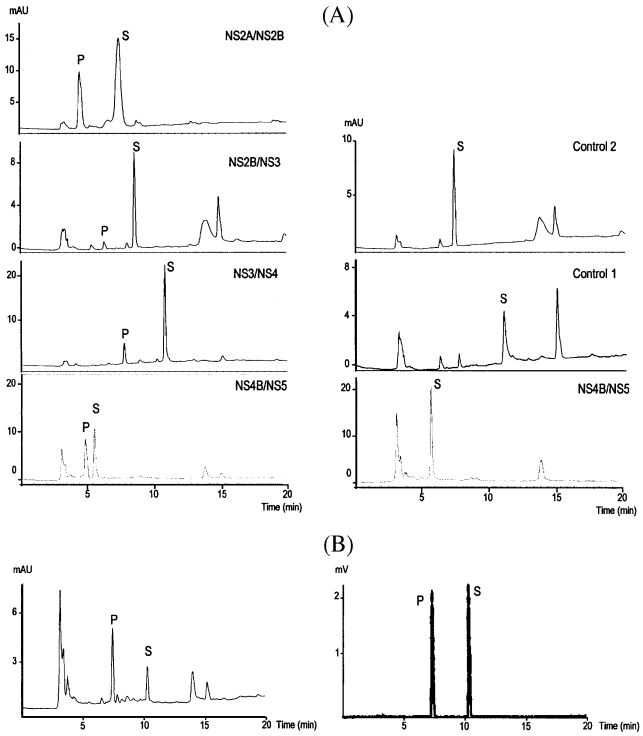

Fig. 3. Reversed-phase HPLC analysis of dansylated peptide substrates that are cleaved by the DEN NS3 protease. All of the samples were run on a C_{18} column, and eluted with a linear gradient of acetonitrile that contained 0.1% (by vol.) trifluoroacetic acid. Assay mixtures contained 10 μM of the peptide substrate in a 100 mM Tris-HCl buffer, pH 8.0, and 2-4 μg of purified NS2B-NS3(pro) or mutant protease (NS2B-NS3(pro)M). The reactions were incubated at 25ºC for 4 h. Panel A shows the UV chromatogram of the hydrolyzed peptide substrates that were detected at 280 nm. Assay mixtures contained the NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 cleavage substrates. For the NS4B/NS5 substrate, a blank control without added enzyme is shown. Control 1 consists of a monobasic NS3/NS4A peptide with a K → A substitution at the P1 residue, and control 2 of a 6mer cleavage site peptide of the NS3/NS4A site. Signals that were generated by substrate peptides and cleavage products are labeled S and P, respectively. Panel B shows the UV chromatogram and the fluorescence signals (λ_{EX} = 340 nm; λ_{EM} = 510 nm) for the cleavage of the NS3/NS4A substrate peptide by recombinant NS2B-NS3(pro). The NS3/NS4A cleavage assay in panel B was incubated for 8 h.

was incubated with the cleavage site peptides that were separated from the reaction products by reversed-phase HPLC. By using UV and fluorescence detection, a decrease of the substrate peak and a concomitant increase of the product signal was observed upon incubation of the substrates with NS2B-NS3(pro). The fluorescence signal of the purified peptide substrates consisted of a single peak, indicating that fluorescent contaminates were not present in the substrate peptide preparations. However, signals of impurities were occasionally observed in the UV chromatograms at retention times between 13 and 16 min. Fig. 3 shows the HPLC separation profiles for the specific cleavage of the synthetic peptide substrates by recombinant NS2B-NS3(pro) protease. Sequences and retention times of the peptide substrates are given in Table 1. In all of the cases, only a single peak of the product appeared after incubation of NS2B-NS3(pro) with the substrates, whereas the control reaction with trypsin produced multiple signals according to the presence of at least two basic residues in the substrates. A 6mer cleavage product peptide FAAGRK of the NS3/NS4A polyprotein site was used as a retention time marker for the HPLC analysis. The cleavage peptide that was produced in NS3-assays that contained the NS3/NS4A substrate co-eluted with this reference peptide in the HPLC separation. Incubation of the NS2B-NS3(pro) protein with a control peptide, FAAGRASLTLNL, containing a lysine to alanine substitution (K2093A) at the P1 position of the NS3/NS4A cleavage site, gave no detectable product that corresponded to the NS3/NS4A cleavage peptide. This is consistent with a strict requirement of the NS3 protease for a basic residue at this position.

The assay has been employed to determine the specific activity of the purified NS2B-NS3(pro) protein. The rates of the cleavage product formation indicate the order NS3/NS4A >NS2A/NS2B>NS4B/NS5>>NS2B/NS3 for the individual NS3 protease cleavage sites (Table 1).

In order to exclude the possibility that co-purification of a

Table 1. Sequences of the cleavage site peptides that encompassed the P6 to P6' residues that were derived from the dengue polyprotein and rates of hydrolysis were observed with purified NS2B-NS3(pro). Amino acid positions refer to the sequence of the DEN 2 strain 16681 (Kinney *et al.*, 1997). Bold triangles (▼) indicate the NS3 cleavage sites in the native polyprotein sequence. Retention times of the substrates and cleavage products are shown.

Cleavage sites	Peptide sequence	Residues	Retention time (min)		Activity (nmol mg^{-1} min^{-1})
			Substrate peptides	Product peptides	
NS2A-NS2B	RTSKKR▼ SWPLNE	1340-1351	7.2	4.4	5.93
NS2B-NS3	EVKKQR▼ AGVLWD	1470-1481	8.4	4.4	0.67
NS3-NS4A	FAAGRK▼ SLTLNL	2088-2099	10.2	7.4	6.83
NS4B-NS5	TTSTRR▼ GTGNIG	2486-2497	5.5	5.0	1.63
Control 1	FAAGRASLTLNL	2088-2099	10.9	-	-
Control 2	FAAGRK	2088-2093	7.4	-	-

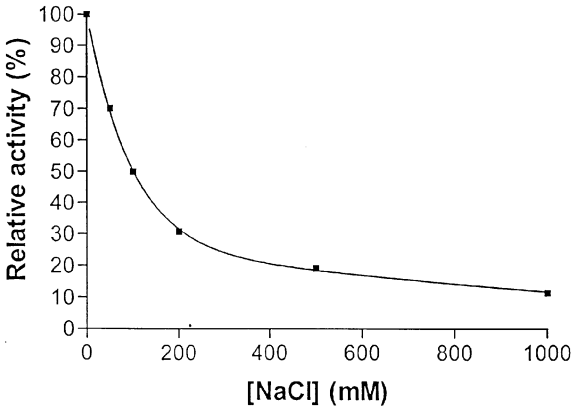

Fig. 4. The effect of salt concentration on the cleavage activity of NS2B-NS3(pro). Activity was measured by hydrolysis assay of the NS3/NS4A substrate peptide in the presence of increasing concentrations of NaCl for 4 h at 25°C.

bacterial protease with a similar specificity was responsible for the proteolysis that we observed with the dibasic peptide substrates, an enzymatically inactive mutant of NS3 was generated by introducing a S135A substitution into the NS3 sequence. The inactive mutant protein was expressed at levels that are comparable to the wild-type enzyme. Pure NS2B-NS3(pro)M protein was prepared under equivalent conditions. Control reactions that contained NS2B-NS3(pro)M showed absolutely no proteolytic activity after incubation up to 8 h with all substrates. This, therefore, confirms that the preparations of NS2B-NS3(pro) were free of contaminating bacterial proteases.

Activity of the enzyme was dependent on the salt concentration. Approximately a 50% decrease of activity was observed in the presence of 0.1 M NaCl (Fig. 4). These data agree with a report that was recently published by Yusof *et al.* (2000) who found a 40% decrease of activity at 0.1 M NaCl concentration (Yusof *et al.*, 2000). The dependence of the NS3 protease activity on the ionic strength of the assay buffer points to a role for electrostatic interactions in the substrate binding by the NS3 enzyme.

Autoproteolytic processing at the NS2B/NS3 site was demonstrated with a fusion protein NS2B(H)-NS3(pro) after purification and refolding from recombinant *E. coli* (Yusof *et al.*, 2000). In contrast, we were unable to detect the products of autocatalytic processing with a renatured NS2B-NS3(pro) protein in a SDS-PAGE analysis. Although dispensable for NS3 protease activity *in vitro*, the hydrophobic flanking regions of NS2B are likely required for targeting the NS3 protease to the membranes of the endoplasmic reticulum *in vivo*. Mutational analysis of polyprotein cleavage provided proof that cleavage at the NS2B/NS3 junction is not a prerequisite for the processing of the downstream cleavage sites (Chambers *at al.*, 1995). Comparative investigation of the enzymatic activities of the NS2B(H)-NS3(pro) and the NS2B-NS3(pro) proteases is required in order to answer the question of whether or not they exhibit different activities *in vitro*.

The NS3(pro) molecule shows higher activity towards small model substrates for serine proteases that contain only one Arg residue as compared to the NS2B(H)-NS3(pro) complex (Yusof *et al.*, 2000). Preferences observed with different types of substrates may reflect significant differences in the specificity pocket of the NS2B-NS3 complex and the unliganded form of the NS3 protease. In the crystal structure of the dengue virus NS3(pro) complex with a Bowman-Birk inhibitor, redundant interactions of the P1 (Arg/Lys) residue of the inhibitor (with residues Asp129, Tyr150, and Ser163 in the NS3 protease) were observed (Murthy *et al.*, 2000). Binding of the NS2B(H) cofactor may mediate enzyme-substrate interactions that extend beyond the S1 subsite of the substrate binding pocket. Based on the crystallographic structure of the DEN 2 NS3(pro) protein (Krishna Murthy *et al.*, 1999), interactions of the substrate side chains with the unliganded enzyme do not appear to extent beyond the P2 and P2 positions. However, the NS2B-NS3 co-complex may contain an expanded substrate binding site that provides additional interactions for substrate recognition. Our assay uses 12mer peptide substrates that mimic authentic polyprotein cleavage sites rather than short tripeptides, which likely satisfy all

enzyme-substrate interactions in the NS2B-NS3 complex. It is, therefore, conceivable that the activities that we observed are reflective of *in vivo* polyprotein processing, where additional enzyme-substrate interactions are involved. We are currently working on the collection of kinetic data for the enzymatic cleavage of the substrate peptides in order to identify substrate preferences for the NS3 protease.

The use of synthetic peptides allows the systematic variation of the substrate sequence to identify residues that are sensitive to substitution. In the absence of a 3-dimensional structure of the NS2B-NS3 complex, these investigations may be useful to define a minimum substrate length for the NS2B-NS3 protease complex in order to analyze residues that are critical for the enzyme-substrate interaction, and to evaluate inhibitors of the DEN NS3 protease.

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