The protease has been shown to form a heterodimeric complex with the small 14 kDa protein NS2B (Falgout *et al.*, 1991), which acts as cofactor for NS3 activation. A region critical for activation of NS3 has been mapped to a 40 amino acid residues charged central domain within NS2B of DEN 4 (Falgout *et al.*, 1993). A central region within the hydrophilic domain of NS2B shows some sequence homology to the NS4A activating peptide of the HCV NS3 protease and molecular modelling has suggested that this subdomain encompassing the sequence G-S-S-P-I-L-I-S-I-T-E could be sufficient for activation of the NS3 protease (Brinkworth *et al.*, 1999).

The crystal structure of the dengue virus NS3 protease domain has been resolved at 2.1 A resolution (Krishna Murthy *et al.*, 1999). The overall conformation of the DEN 2 NS3 N-terminal domain without 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 has been 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 2B/3 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. Presence of NS2B(H) resulted in a several thousand-fold activation of the NS3 protease towards dibasic peptide substrates. In contrast, 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 have provided 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 have 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 fused to a N-terminal polyHis affinity tag by using fluorescence-labeled peptides representing native dengue polyprotein cleavage sequences.

MATERIALS AND METHODS

Plasmids and site-directed mutagenesis

The DNA sequence encoding 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 template. The DNA segment was cloned into the

expression vector pTrcHisA (Invitrogen, San Diego, USA) and transformed into *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, LaJolla, USA). Complementary mutagenic oligonucleotide primers 5'-CCTGGAACTGCAGGATCTCCAATTATCG-3' and 5'- CGATAATTGGAGATCCTGCAG-TTCCAGG-3' introducing simultaneously a *Pst*I site suitable for restriction screening were purchased from Genset Inc., Singapore and PCR reactions were run on a thermal cycler Gene Amp System 2400 (Perkin Elmer, Norwalk, USA). The sequence of the mutated NS3(pro) gene was verified by DNA sequencing using a Perkin Elmer ABI prism 377 sequencer (Perkin Elmer, Norwalk, USA).

Purification of NS2B-NS3(pro)

The recombinant NS2B-NS3(pro) and NS2B-NS3(pro)M proteins of dengue virus type 2 were expressed as inclusion bodies in bacterial cells *E. coli* C41 and purified by Ni^{2+} - chelate affinity chromatography. The protein was refolded by stepwise dialysis against 4 changes of 300 mM NaCl, 100 mM Tris-HCl, pH 8.0, for 20 hrs at 4°C to remove urea. The presence of the NS2B-NS3(pro) protein was detected in Coomassie-blue stained SDS-PAGE gels as well as by Western blotting on nitrocellulose filters using an anti-histidine affinity tag antiserum (Invitrogen, San Diego, USA). Band intensities of proteins were analyzed by densitometry using a Bio-Rad Geld Doc 1000 gel imaging system. Protein concentrations in samples were determined by using a Bio-Rad protein quantitation kit with BSA as a standard. Purified protease preparations were stored in 150 mM NaCl, 40% glycerol, 50 mM Tris-HCl, pH 8.0 at -20°C. Trypsin (Sigma Chemistry, St. Louis, USA) was used as positive control for cleavage assays.

Peptide Substrates

12mer peptide substrates encompassing the native dengue virus type 2 strain 16681 polyprotein 2A/B, 2B/3, 3/4A and 4/B cleavage sites containing a N-terminal dansyl group (Dns) were commercially synthesized by Biosynthesis Inc., Lewisville, USA., and purified by reverse-phase HPLC. A 6mer cleavage product peptide Dns-FAAGRK of the 3/4A site was used as retention reference for HPLC analysis. The monobasic peptide Dns-FAAGRASLTLNL containing a K→A substitution at the P1 position served as negative control in cleavage assays with DEN NS3 protease. Peptides were solubilized in xx.

HPLC DEN NS3 Protease Assays

Reaction mixtures for the detection of NS3 proteolytic activity were composed of 100 mM Tris-HCl, pH 8.0, containing 10 μM 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. Samples were incubated at room temperature (25°C) for various periods of time and reactions were quenched by the addition of acetonitrile to give a final concentration of 20%. Samples were loaded on a reversed-phase

HPLC (Waters, model X) and separated on a C-18 phenomenex column (250 mm x 4.6 mm, Pharmacia) equilibrated with 0.1% (vol./vol.) TFA/water. Reaction products were separated by a linear gradient of 2% min^{-1} of 0.1% TFA/acetonitrile. Products were analyzed by UV-detection at 214 and 280 nm and by a fluorescence monitor (Waters 474) set to excitation wavelength λ = 340 nm and emission wavelength λ = 510 nm. The extend of hydrolysis was estimated from the decrease in area of the substrate peak. Injection of standard amounts of substrate was used to calibrate peak areas.

RESULTS AND DISCUSSION

A number of reports in the 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; Wang *et al.*, 1997; Seybert *et al.* 1997). In analogy to these procedures, we have used a series of synthetic peptides representing authentic proteolytic cleavage sites of dengue virus serotype 2 as substrates to analyze the enzymatic activity of a purified NS2B-NS3(pro) protease complex *in vitro*.

Dengue virus NS2B-NS3(pro) fused to a N-terminal 6xHis purification tag was purified from overexpressing *E. coli* C41 harbouring the pTrcHis/2B-3(pro) expression plasmid (Figure 1). The NS2B-NS3(pro) protein was recovered from inclusion bodies after solubilization in 8M urea and purified by Ni^{2+} - metal affinity column chromatography under denaturing conditions. The purified protein was refolded by stepwise dialysis. Figure 2 shows a SDS-PAGE gel of a representative purification and the corresponding Western blot of the gel probed with anti-polyhistidine antiserum. A major immunoreactive band at 35 kDa molecular weight was observed following elution from the affinity column in the presence of 0.5 M imidazole. By comparison of band intensities on Coomassie blue - stained gels, the amount of NS2B-NS3(pro) produced per liter of *E. coli* culture grown to OD_{600}=1.0 was estimated to 1 mg. NS2B-NS3(pro) was recovered at a purity of >95% as estimated from 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 the sequences corresponding to the 2A/B, 2B/3, 3/4A and 4B/5 cleavage sites of the DEN 2 polyprotein were chemically synthesized and N-terminally labeled with a dansyl group to facilitate photometric detection by UV as well as fluorescence detection. 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. Purified NS2B-NS3(pro) was incubated with the cleavage site peptides which were separated from the reaction products by reverse-phase HPLC. By using 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). In all cases, only a single peak of 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. Figure 3 shows the HPLC separation profiles for the specific cleavage of the synthetic peptide substrates by recombinant NS2B-NS3(pro) protease. Retention times for substrates and products observed under the conditions employed are given in table 1. A 6mer cleavage product peptide FAAGRK of the 3/4A polyprotein site was synthesized and used as retention time marker for HPLC analysis. The cleavage peptide produced in NS3-assays containing the 3/4A substrate co-eluted with this reference peptide in the HPLC separation. Incubation of the NS2B-NS3(pro) protein with a control peptide, F-A-A-G-R-A-S-L-T-L-N-L, containing a K→A substitution at the P1 position of the 3/4A cleavage 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 (Figure 4).

Substrate peaks were used to calibrate the amounts of peptides consumed by the enzymatic reaction and relative rates of hydrolysis were determined under identical conditions for the cleavage site peptides representing the 2A/B, 2B/3, 3/4A and 4B/5 cleavage sites (Table 1). The activity observed for the 3/4A cleavage site was found to be the highest for all peptides and the 2B/3 cleavage site showed the lowest activity.

Autoproteolytic processing at the 2B/3 site has been demonstrated with a fusion protein NS2B(H)-NS3(pro) after purification and refolding from recombinant *E. coli* (Yusof *et al.*, 2000). In contrast, we have not been able to detect the products of autocatalytic processing with the NS2B-NS3(pro) protein in 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 has provided evidence that cleavage at the 2B/3 junction is not a prerequisite for 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 to answer the question whether they exhibit different activities *in vitro*.

The NS3(pro) molecule shows higher activity towards small model substrates for serine proteases containing only one Arg residue than 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 have been observed (Krishna Murthy *et al.*, 2000). Binding of the NS2B(H) cofactor was suggested to mediate enzyme-substrate interactions extending beyond the S1 subsite of the substrate binding pocket. Based on the crystallographic structure of the DEN NS3(pro) (Krishna Murthy *et al.*, 1999), interactions of the substrate side chains with the unliganded enzyme appear not to extent beyond the P2' and P2 positions, whereas the NS2B-NS3 co-complex may contain an expanded substrate binding site which provides additional interactions for substrate recognition. Our assay uses 12mer peptide substrates mimicking authentic polyprotein cleavage sites rather than short tripeptides. The 12mer peptides likely satisfy all enzyme-substrate interactions in the NS2B-NS3 complex. It is therefore conceivable that the activities we have observed are reflective of *in vivo* polyprotein processing where additional enzyme-substrate interactions are involved.

The use of synthetic peptides allows the systematic variation of the substrate sequence to identify residues which 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, to analyze residues critical for enzyme-substrate interaction and to evaluate inhibitors of the DEN NS3 protease.

ACKNOWLEDGEMENTS

We thank S. Leetachewa for technical assistance and Dr. Duncan R. Smith for critical reading of the manuscript. This work is supported by grants from the National Science and Technology Development Agency (NSTDA) and the Thailand Research Fund (TRF).

REFERENCES

Arias, C. F., Preugschat, F., 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.

Bhamarapravati, N. and Sutee, Y. (2000) Live attenuated tetravalent dengue vaccine. Vaccine 18, 44-47.

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.

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

Chambers, T. J., Nestorowitz, A. and Rice, C.M. (1995) Mutagenesis of the yellow fever virus NS2B/3 cleavage site: determinants of cleavage site specificity and effects on polyprotein processing and viral replication. J. Virol. 69, 1600-1605.

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.

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.

Falgout, B. and Markoff, L. (1995) Evidence that flavivirus NS1-NS2A cleavage is mediated by a membrane-bound host protease in the endoplasmic reticulum. J. Virol. 69, 7232-7234.

Gubler, D.J. and Clark, G.G. (1995) Dengue / dengue hemorrhagic fever: the emergence of a global health problem. Emerg. Infect. Dis. 1, 55-57

Huang, C. Y., Butrapet, S., Pierro, D. J., Chang, G.J., Hunt, A.R., Bhamarapravati, N., Gubler, D. J. and Kinney, R. M. (2000) Chimeric dengue type 2 (vaccine strain PDK-53) / dengue type 1 virus as a potential candidate dengue type 1 virus vaccine. J. Virol. 74, 3020-3028.

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

Kakiuchi, N., Komoda, Y., Hijikata, M. and Shimotohno K. (1997) Cleavage activity of hepatitis C virus serine protease. J. Biochem. 122, 749-755

Kim, J. L., Morgenstern, K. A., Lin, C., Fox, T., Dwyer, M. D., Landro, J. A., Chambers, S. P., Markland, W., Lepre, C. A., O'Malley, 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.

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

Krishna Murthy, H. M., Judge, K., DeLucas, L. and Padmanabhan, R. (2000) Crystal structure of dengue virus NS3 protease in complex with a Bowman-Birk inhibitor: implications for flaviviral polyprotein processing and drug design. J. Mol. Biol. 301, 759-767.

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.

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.

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.

Markoff, L. (1989) In vitro processing of dengue virus structural proteins: cleavage of the pre-membrane protein. J. Virol. 63, 3345-3352.

Monath, T. P. (1994) Dengue: the risk to developed and developing countries. Proc. Natl. Acad. Sci. USA 91, 2395-2400.

Nowak, T., Farber, T.M., Wengler, G. and Wengler, G. (1989) Analyses of the terminal sequences of West Nile virus structural proteins and of the in vitro translation of these proteins allow the proposal of a complete scheme of the proteolytic cleavages involved in their synthesis. Virology, 169, 365-376.

Rigau-Perez, J.G., Clark, G.G., Gubler, D.J., Reiter, P., Sanders, E.J. and Vorndam, A.V. (1998) Dengue and dengue hemorrhagic fever. Lancet 352, 971-976.

Seybert, A., Ziebuhr, J. and Siddel, S. G. (1997) Expression and characterization of a recombinant murine coronovirus 3C-like proteinase. J. Gen. Virol. 78, 71-75.

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.

Wang, Q. M., Sommergruber, W. and Johnson, R. B. (1997) Cleavage specificity of human rhinovirus-2 2A protease for peptide substrates. Biochem. Biophys. Res. Commun. 235, 562-566.

Yusof, R., Clum, S., Wetzel, M., Krishna Murthy, H. M. and Padmanabhan, R. (2000) Purified NS2B/NS3 serine protease of dengue virus type 2 exhibits cofactor NS2B dependence for cleavage of substrates with dibasic amino acids in vitro. J. Biol. Chem. 275, 9963-9969.

Table 1

POLYPROTEIN JUNCTION	PEPTIDE SEQUENCE	REL. HYDROLYSIS (%)
NS2A / B	RTSKKR∇SWPLNE	65
NS2B / 3	EVKKQR∇AGVLWD	< 20
NS3 / 4A	FAAGRK∇SLTLNL	100
NS4A / 5	TTSTRR∇GTGNIG	86
CONTROL 1	FAAGRASLTLNL	< 1
CONTROL 2	FAAGRK	-

Table 1: Peptide substrates for the DEN polyprotein junctions and relative rates of hydrolysis observed for cleavage by the NS3 serine protease.

TITLES TO FIGURES

Fig. 1

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

Fig.2

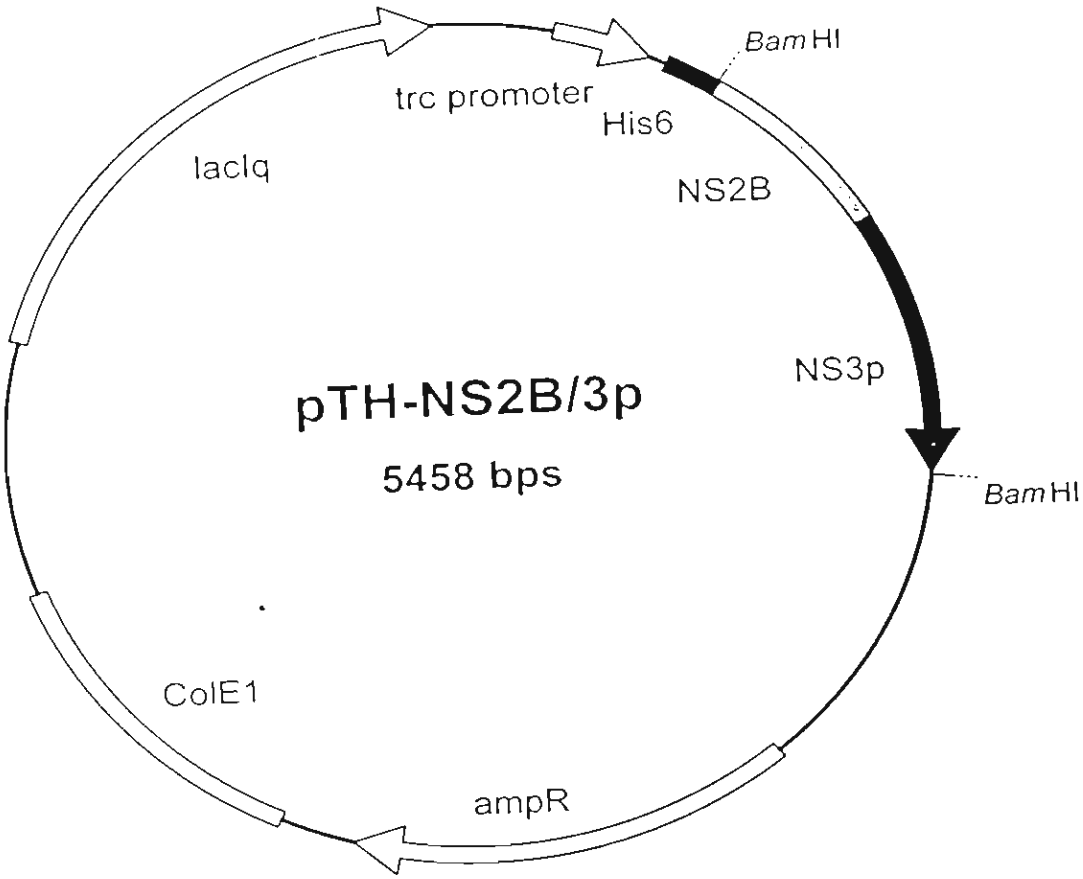
Coomassle-stained 12.5% SDS-PAGE gel analysis of DEN NS2B-NS3(pro) protease purification. Lane 1: molecular weight marker; lane 2: solubilized inclusion bodies from *E. coli*; lane 3: sepharose Q fractions; lane 4: eluant from metal affinity column in the presence of 0.5 M imidazole. M indicates the position of the marker proteins in kDa.

Fig. 3

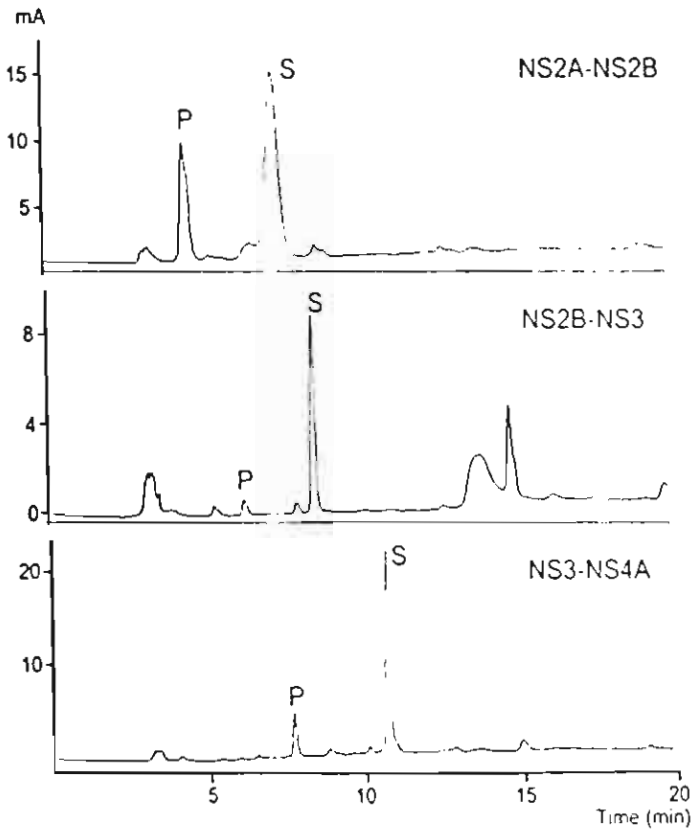
Reverse-phase HPLC analysis of dansylated peptide substrates cleaved by DEN NS3 protease. All samples were run on a C_{18} column and eluted with a linear gradient of acetonitrile containing 0.1% (by vol.) trifluoroacetic acid. Assay mixtures contained 10 mM of the peptide substrate in 100 mM Tris-HCl buffer, pH 8.0, and 2 - 4 μg of purified NS2B-NS3(pro) or mutant protease (S135A). Reactions were incubated at 25°C for 8 h. Assay mixtures were composed of a) 2A/B, b) 2B/3, c) 3/4A and d) 4B/5 cleavage substrates, respectively. Peptides were detected by UV (214 nm) and fluorescence (λ_{EX} = 340 nm; λ_{EM} = 510 nm).

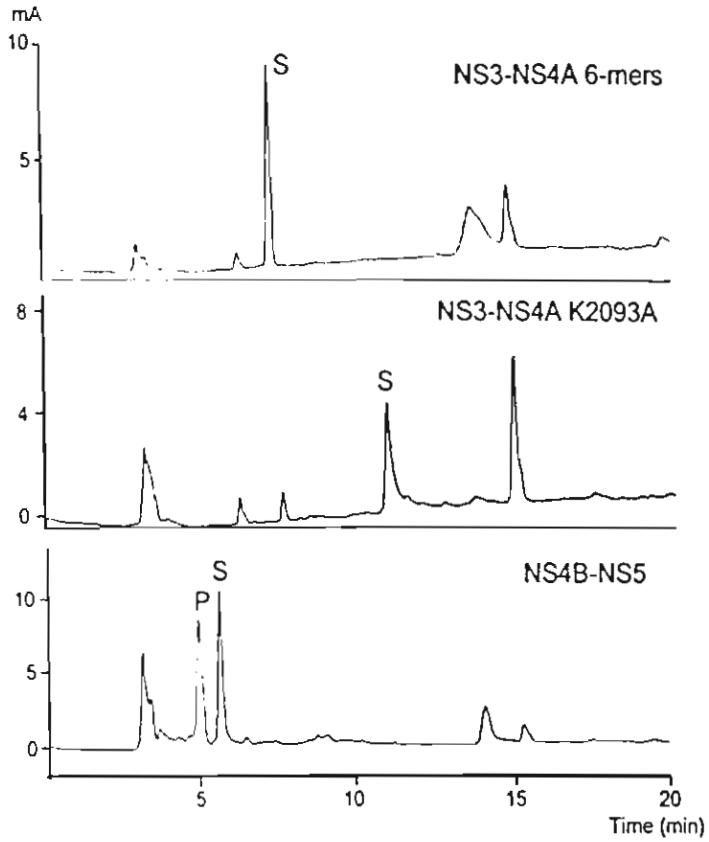
Fig. 4

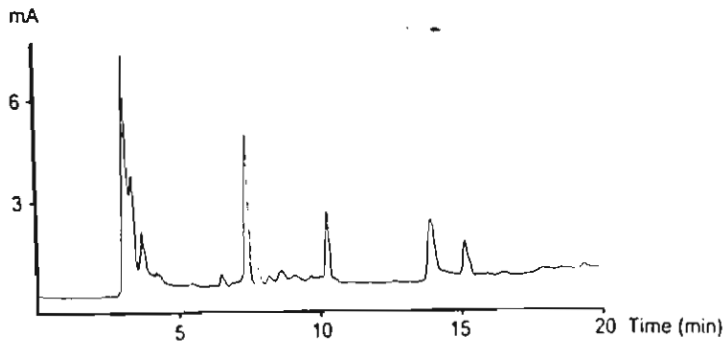
Effect of salt concentration on cleavage activity of NS2B-NS3(pro). Activity was measured by hydrolysis assay of the 3/4A substrate peptide in the presence of increasing concentrations of NaCl for 4 h at 25°C.

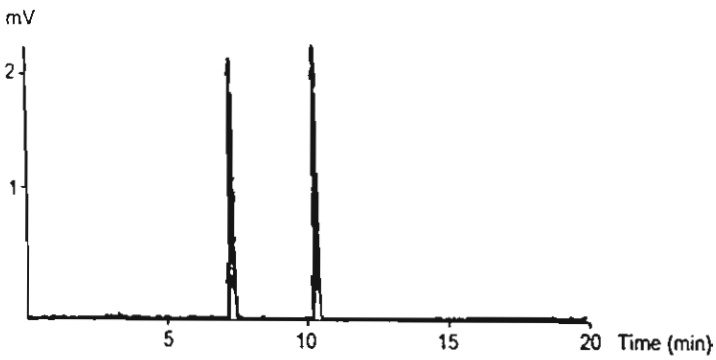

BamHI
trc promoter
His6
lacIq
NS2B
NS3p
pTH-NS2B/3p
5458 bps
BamHI
ColE1
ampR

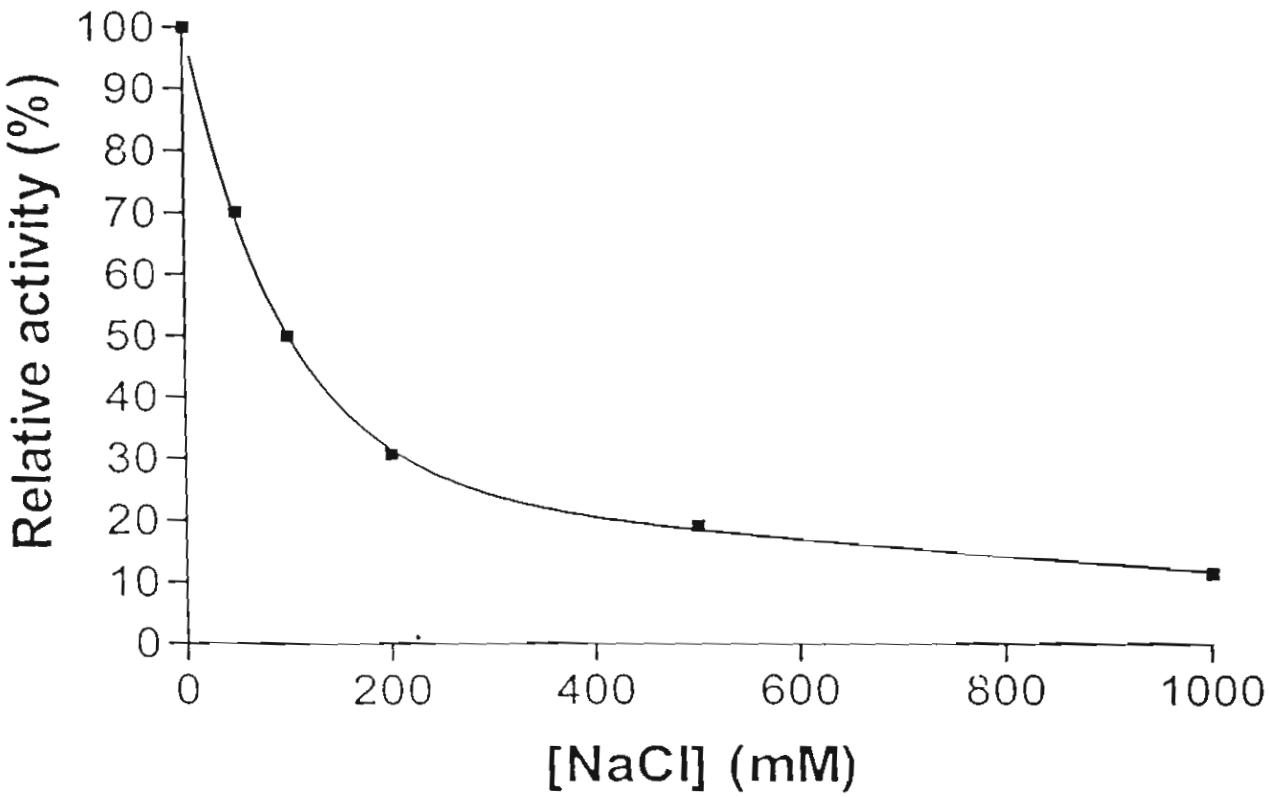
1 - low range molecular weight marker
2 - inclusion bodies
3 - NS2B-NS3p purified by Hi-Trap chelating 1 ml
4 - NS2B-NS3p purified by Superdex200 HR10/30


mA
NS2A-NS2B
S
P
NS2B-NS3
NS3-NS4A
Time (min)


mA
NS3-NS4A 6-mers
NS3-NS4A K2093A
NS4B-NS5
S
P
Time (min)


mA
Time (min)


mV
Time (min)


Relative activity (%)
100
90
80
70
60
50
40
30
20
10
0
0
200
400
600
800
1000
[NaCl] (mM)