Enzymatic Analysis of Recombinant Japanese Encephalitis Virus NS2B(H)-NS3pro Protease with Fluorogenic Model Peptide Substrates

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Abstract

Background: Japanese encephalitis virus (JEV), a member of the Flaviviridae family, causes around 68,000 encephalitis cases annually, of which 20–30% are fatal, while 30–50% of the recovered cases develop severe neurological sequelae. Specific antivirals for JEV would be of great importance, particularly in those cases where the infection has become persistent. Being indispensable for flaviviral replication, the NS2B-NS3 protease is a promising target for design of anti-flaviviral inhibitors. Contrary to related flaviviral proteases, the JEV NS2B-NS3 protease is structurally and mechanistically much less characterized. Here we aimed at establishing a straightforward procedure for cloning, expression, purification and biochemical characterization of JEV NS2B(H)-NS3pro protease.

Methodology/Principal Findings: The full-length sequence of JEV NS2B-NS3 genotype III strain JaOArS 982 was obtained as a synthetic gene. The sequence of NS2B(H)-NS3pro was generated by splicing by overlap extension PCR (SOE-PCR) and cloned into the pTrcHisA vector. Hexahistidine-tagged NS2B(H)-NS3pro, expressed in E. coli as soluble protein, was purified to >95% purity by a single-step immobilized metal affinity chromatography. SDS-PAGE and immunoblotting of the purified enzyme demonstrated NS2B(H)-NS3pro precursor and its autocleavage products, NS3pro and NS2B(H), as 36, 21, and 10 kDa bands, respectively. Kinetic parameters, $K_m$ and $k_{cat}$ for fluorogenic protease model substrates, Boc-GRR-amc, Boc-LRR-amc, Ac-nKRR-amc, Bz-nKRR-amc, Pyr-RTKR-amc and Abz-(R)4SAG-nY-amide, were obtained using inner filter effect correction. The highest catalytic efficiency $k_{cat}/K_m$ was found for Pyr-RTKR-amc ($k_{cat}/K_m$: 1962.96 ± 85.0 M$^{-1}$ s$^{-1}$) and the lowest for Boc-LRR-amc ($k_{cat}/K_m$: 3.74 ± 0.3 M$^{-1}$ s$^{-1}$). JEV NS3pro is inhibited by aprotinin but to a lesser extent than DEN and WNV NS3pro.

Conclusions/Significance: A simplified procedure for the cloning, overexpression and purification of the NS2B(H)-NS3pro was established which is generally applicable to other flaviviral proteases. Kinetic parameters obtained for a number of model substrates and inhibitors, are useful for the characterization of substrate specificity and eventually for the design of high-throughput assays aimed at antiviral inhibitor discovery.

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Introduction

Japanese encephalitis virus (JEV) is a mosquito borne flavivirus that causes severe central nervous system diseases such as an acute flaccid paralysis, aseptic meningitis and encephalitis [1]. It belongs to the genus Flavirus (family Flaviviridae) which comprises over 70 viruses many of which are human pathogens, including West Nile virus (WNV), Dengue virus (DEN), Yellow fever virus (YFV), Murray Valley Encephalitis Virus (MVEV), Kunjin Virus (KUNV) and Tick-Borne Encephalitis Virus (TBEV) [2]. Japanese encephalitis (JE) is among the most important viral encephalitides in Asia [3–5]. Moreover, the disease is not restricted to Asia and cases also occur sporadically in northern Australia and western Pacific [6]. Of about 68,000 estimated annual cases, approximately 20–30% are fatal, and 30–50% of survivors have significant neurologic sequelae [7,8]. Since the zoonosis is endemic in large parts of Asia, it is not likely to ever be extinguished.

Currently there is no antiviral therapy for JEV or any other flaviviral infection, and so far the main strategy to control the incidence is by preventive methods such as vaccination and preventing mosquito bites [9–11]. Although the improvements in JEV vaccination coverage has reduced the JE incidence, about 55,000 (81%) out of the total annual cases still occur in areas with well established or developing JE vaccination programs [12].
Effective antiviral therapy is thus urgently needed, especially for those cases where the infection has become persistent. One approach to develop anti-JEV therapy is to interfere with the life cycle of the virus, and exploit the molecular targets such as envelope glycoprotein, NS3 protease, NS3 helicase, NS5 methyltransferase and NS5 RNA-dependent RNA polymerase [13]. Unlike several other flaviviruses such as DEN, WNV and MVEV [14–16] whose protease enzymes are extensively characterised as potential drug targets, the JEV protease is comparatively less studied with a view to structure-activity relations.

The JEV two-component protease NS2B/NS3 is responsible for processing the viral polyprotein precursor to the mature viral proteins involved in viral pathogenesis, and therefore considered an important drug target in JEV [17,18]. The N-terminal one-third (180 residues) of NS3 represents the protease domain NS3(pro) that works in coordination with the C-terminal two-third portion RNA helicase during viral propagation [19,20]. The proteolytic domain contains a classical catalytic triad of H51, D75 and S135, and autocatalytic proteolytic cleavage at the NS2B/NS3 polypeptide junction leads to the formation of a non-covalent complex of NS2B and NS3 [21]. Earlier studies have revealed that a 35–48 amino acid residues long central hydrophilic region NS2B(H) of NS2B interacts directly with the NS3(pro) and promotes folding of NS3(pro) into a catalytically competent conformation [22–25].

Currently, there is no X-ray crystallographic structure available for the JEV protease, but crystal structures of the similar proteases from DEN and WNV have provided insight into the mechanism of cofactor-dependent activation and revealed an ‘induced fit’ mechanism of catalysis [26,27]. By analysis of chimeric viral proteases of DEN2 and YFV, it was shown that the YFV polypeptide cleavage sites were efficiently cleaved by the chimeric protease containing the YFV or DEN2 NS3 protease domain, while the DEN2 polypeptide sites were not cleaved by the YFV chimeric protease containing YFV NS3(pro), suggesting that cleavage requires specific local interactions between substrates and the binding pocket site of the enzyme [28]. The substrate recognition sequence is highly conserved in all flaviviruses and consists of two basic residues in P2 and P1 followed by a small unbranched amino acid in P1′ [22,43]. Substrate profiling studies found that the WNV protease was highly selective for the cleavage site sequence motif (K/R)↓G, whereas DEN protease also tolerated the presence of bulky residues such as Phe, Trp, or Tyr at the position of Gly [21,28,29].

The aim of this study was to develop a fast and easy methodology for cloning, expression and purification of the active JEV NS2B(H)-NS3 serine protease and to obtain numerical data for kinetic constants by using urorogenic model peptide substrates for serine proteases. In addition, we also characterized inhibition of the protease by conventional serine protease inhibitors. To the best of our knowledge, this is the first study into the biochemical characteristics, substrate preferences and inhibitor profile of the protease encoded in the JEV genome.

Materials and Methods

Construction of JEV NS2B(H)-NS3pro Expression Plasmid

The full-length sequence of JEV genotype III strain JaOArS89 982 NS2B-NS3 (Genebank accession number: M18370) was custom-synthesized (Blue Heron Technology Inc., Canada) and cloned into pLS vector (Top Gene Technologies, Canada). In close analogy with procedures previously published for DEN NS2B(H)-NS3pro [30], the pLS/NS2B-NS3 plasmid template was used to PCR-amplify the NS2B(H) region (amino acids 51–95 and 121–131 using specific primers NS2B(H)-F (5′-GGATCCGTTGCTAGAAAGCAACAGATATGGCTTGAACTGACGAGC-3′, (underlined sequence represents the BamHI restriction site) and NS2B(H)-R (5′-GGCCCTCTTTTTGTGTTTCTAAATGGAGCTGACGAGCTACCTTTTCTGAAAAACAAAAAGAAAAGGGGCGTGTTTTTGGAACCCGC-3′, (underlapping sequence in bold letters); and NS3(pro)-F (5′-GGCGGTGTTCAGTATTGGCTCAGCTTTTTCTGAAAAACAAAAAGAAAAGGGGCGTGTTTTTGGAACCCGC-3′, (underlapping sequence in bold letters); and NS3(pro)-R: 5′-GGTTATTGGCTCACTTTTGAAGCTGTCGACCTGACACACAGGAG-3′) into pLS vector (Top Gene Technologies, Canada). In close analogy with procedures previously published for DEN NS2B(H)-NS3pro [14–16] whose protease enzymes are extensively characterised as potential drug targets, the JEV protease is comparatively less studied with a view to structure-activity relations.

The JEV two-component protease NS2B/NS3 is responsible for processing the viral polyprotein precursor to the mature viral proteins involved in viral pathogenesis, and therefore considered an important drug target in JEV [17,18]. The N-terminal one-third (180 residues) of NS3 represents the protease domain NS3(pro) that works in coordination with the C-terminal two-third portion RNA helicase during viral propagation [19,20]. The proteolytic domain contains a classical catalytic triad of H51, D75 and S135, and autocatalytic proteolytic cleavage at the NS2B/NS3 polypeptide junction leads to the formation of a non-covalent complex of NS2B and NS3 [21]. Earlier studies have revealed that a 35–48 amino acid residues long central hydrophilic region NS2B(H) of NS2B interacts directly with the NS3(pro) and promotes folding of NS3(pro) into a catalytically competent conformation [22–25].

The sequence of the recombinant (His)6-NS2B(H)-NS3pro construct was confirmed by DNA sequence analysis (Macrogen, South Korea), using the sequencing primers, pTrHis-F: 5′-GGAGGTATATATAAATGTAACG-3′; and pTrHis-R: 5′-CTGAAAATCTTCTTCTACATCG-3′.

Expression and Purification of NS2B(H)-NS3pro

Escherichia coli DH5α (GIBCO BRL, USA) was used as host cell for plasmid propagation. E. coli C41 (F′, ompT, hsdSB, (rB− mB−), gal, den, (DE3)), derived from E. coli C41 (DE3), was used as expression host. Constructs were transformed into E. coli (BL21) and cells were grown in one liter LB medium containing 100 μg ml−1 ampicillin, at 37°C, until OD600 reached 0.6. Expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) at a concentration of 0.2 mM and cells were incubated for 15 h at 18°C. Cells were harvested by centrifugation (6000 × g, 4°C, 10 minutes) and the pellet was resuspended in 30 ml lysis buffer.
Industry, Inc. MA, USA), against three batches of a 100-fold volume buffer A (0.1 M Tris-HCl, pH 8.5, 0.2 M NaCl), and the sample volume buffer B (0.1 M Tris-HCl, pH 8.5, 0.3 M NaCl) and the sample (30 ml from 1 liter of bacterial culture) was loaded at a flow rate of 1 ml min⁻¹, using a FPLC pump (AKTA™FPLCM system, GE Healthcare). The column was washed with 10 column volumes of degassed washing buffer (0.1 M Tris-HCl, pH 7.5, 0.3 M NaCl, 30 mM imidazole). Protein was eluted with ten column volumes elution buffer (0.1 M Tris-HCl, pH 7.5, 0.3 M NaCl, 0.3 M imidazole) at flow rate of 1.0 ml min⁻¹. Elution was monitored by absorbance at 280 nm using a UV detector (AKTA™FPLCM system, GE Healthcare) and fractions of 1.0 ml were collected. Aliquots of 20 µl from each fraction were loaded onto a 15% SDS-PAGE gel and electrophoresis was performed in Tris-glycine buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine and 0.1% SDS). The gel was stained with Coomassie-Blue staining solution (0.1% Coomassie-Brilliant-Blue R250, 50% methanol and 10% glacial acetic acid) with shaking at room temperature for 2 hours, and then destained with shaking in destaining solution (10% methanol and 10% glacial acetic acid) at room temperature, overnight. Western blotting was performed using anti-hexahistidine antiserum (Invitrogen, CA, USA) with bovine serum albumin (Sigma Chemistry) as calibration standard. Samples were stored in 50 mM Tris-HCl, pH 9.0, (50% v/v) glycerol, 0.5 mM ac-nKRR-amc and five residues, Pyr-RTKR-amc, and a previously described Boc-LRR-amc; four residues, Ac-nKRR-amc; and two non-prime side residues, Boc-GRR-amc and Pyr-GRR-amc; with exception of the Boc-LRR-amc peptide substrate where SD was 12.5%.

**Results**

3.1. Cloning, Expression and Purification of JEV NS2B(H)-NS3pro

Starting from an in vitro synthesized gene sequence encoding the full-length NS2B-NS3 protein from JEV, an enzymatically active single-chain protease NS2B(H)-NS3pro was constructed by SOE-PCR and cloned downstream of an N-terminal hexahistidine purification tag into expression vector pTrcHisA (Fig. 1). Recombinant plasmid DNA was transformed into E. coli DH5α followed by rapid size screening and restriction digestion analysis. The complete sequence of the cloned JEV NS2B(H)-NS3pro was analyzed by automated DNA sequencing in both...
forward and reverse directions, and resulting sequences were compared to the nucleotide sequence of JEV genotype III strain JaOArS 982 [Genebank accession number M18370.1]. No premature stop codons or amino acid substitutions were introduced in the recombinant sequence. NS2B(H)-NS3pro was expressed upon incubation for 12 h in the presence of 0.2 mM IPTG, predominantly as a soluble protein and was purified to >95% purity by a single-step chromatography on Ni²⁺-metal chelate affinity columns, eluting at 0.3 M imidazole (Fig. 2). SDS-PAGE analysis and subsequent Western immunoblotting with anti-polyhistidine antibodies of the purified protein revealed the presence of two major proteins with molecular weights 21 and 10 kDa, and a relatively faint band at 36 kDa, thereby suggesting almost complete autocleavage of the enzymatically active NS2B(H)-NS3pro protease at the native NS2B/NS3 cleavage site (Fig. 3).

### 3.2 Enzyme Assay with Fluorogenic Peptide Substrates

Enzymatic activity of the recombinant NS2B(H)-NS3pro protein was assayed by fluorescence release from several small synthetic peptide substrates resembling the dibasic cleavage site sequences of the JEV polyprotein precursor. In addition, we have analysed the activity of JEV NS2B(H)-NS3pro by using a tetradic internally quenched fluorescent peptide, Abz-(R)₄SAGnY-amide, originally described for the DEN NS3 serine protease [22]. Peptide substrates labelled with the amc reporter group comprised three to five amino acid residues at the non-prime side, whereas the tetradic substrate contained three residues added to the

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**Figure 1. Schematic representation of primer binding sites and physical map of pTrcHisA/NS2B(H)-NS3(pro).**

- **A** The figure illustrates the NS2B(H)-NS3(pro) fragment and the primer binding positions. The NS2B(H), NS2B C terminal 11 amino acid residues linker and NS3 protease domains are shown. The NS2B-NS3 cleavage site is represented as a triangle. PCR primers are shown in maroon lines representing overlapping sequences. Amino acid positions within NS2B and NS3 are shown as black letters.
- **B** Shown is the recombinant plasmid, pTrcHisA/NS2B(H)-NS3(pro) of JEV encoding the 31 kDa NS2B(H)-NS3(pro) from JEV. The plasmid backbone contains the trc promoter (pTrc), lac operator (lacO), polyhistidine (His₆) tag, Xpress™ epitope (Xpress), ampicillin resistance gene (AmpR) and lacIq repressor genes (lacIq). The plasmid map was generated by the Vector NTI program.

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earlier study on DEN2 NS2B-NS3 protease where values of all amc-labelled peptides. This substrate has been described in an
variations ranging from $K_m$ lowest $YFV$ and DEN2 proteases. 

Cleavage sites of YFV and DEN2 viruses seem to be bound equally 
DEN2 [34,35] Thus, this substrate containing P2-P1 Arg-Arg as in 

fit of data, and kinetic parameters, Michaelis-Menten kinetics, as revealed by non-linear least square 
inner filter effects, data were in excellent agreement with 
a function of substrate concentration (Fig. 4). Upon correction of 
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(0.1 M Tris-HCl, pH 7.5, 0.3 M NaCl, 0.3 M imidazole (peak B). 

The column was washed with 0.1 M Tris-HCl, pH 7.5, 0.3 M NaCl, 30 mM 
imidazole (peak A) and NS2B(H)-NS3pro was eluted with elution buffer 
(0.1 M Tris-HCl, pH 7.5, 0.3 M NaCl, 0.3 M imidazole (peak B). 

 prime side of the peptide cleavage sequence. Initial velocities for 
each substrate (RFU min$^{-1}$) were converted to concentrations of released amc and velocities (nm min$^{-1}$) were re-plotted as 
a function of substrate concentration (Fig. 4). Upon correction of 
inner filter effects, data were in excellent agreement with 
Michaelis-Menten kinetics, as revealed by non-linear least square 
fit of data, and kinetic parameters, $K_m$, $k_{cat}$, and catalytic efficiency 
$k_{cat}/K_m$ were determined for each substrate (Table 1).

Sequences of the peptides analysed showed differing substrate 
binding activities ($K_m$ as well as substrate turnover ($k_{cat}$) and kinetic 
data obtained for the substrates displayed relatively large variations ranging from $k_{cat}/K_m$ 3.74 M$^{-1}$ s$^{-1}$ (Boc-LRR-amc) 
to 1,963 M$^{-1}$ s$^{-1}$ (Pyr-RTKR-amc) to 1,963 M$^{-1}$ s$^{-1}$ (Pyr-RTKR-amc). The most inefficient sub- 
strate (based on $k_{cat}/K_m$) was Boc-LRR-amc, with a low $k_{cat}$ of 
0.00015 s$^{-1}$. Although the second least efficiently cleaved amc- 
labeled substrate, Boc-GRR-amc, showed a 3-fold higher $K_m$ 
(123 μM), turnover was approximately 10-fold greater than for 
Boc-LRR-amc. In earlier studies Boc-GRR-amc has shown $K_m$ of 
142 μM and $k_{cat}$ of 0.034 s$^{-1}$ on NS2B/NS3 protease of YFV and 
$K_m$ of 150 μM and $k_{cat}$ of 0.13 s$^{-1}$ on NS2B/NS3 protease of 
DEN2 [34,35]. Thus, this substrate containing P2-P1 Arg-Arg as in 
cleavage sites of YFV and DEN2 viruses seem to be bound equally 
and cleaved with lower turnover by JEV protease than by 
YFV and DEN2 proteases.

The most efficiently cleaved substrate, Pyr-RTKR-amc, had the 
lowest $K_m$ (9 μM) and the highest turnover number (0.0176 s$^{-1}$) of 
all amc-labelled peptides. This substrate has been described in an 
earlier study on DEN2 NS2B-NS3 protease where values of 
134 μM, 0.013 s$^{-1}$ and 97 M$^{-1}$ s$^{-1}$ were reported for $K_m$, $k_{cat}$ and $k_{cat}/K_m$ respectively [24]. It was also described in another 
study on N2B-NS3 proteases of WNV and DEN2, where the $K_m$ 
values were 71 μM, 6.3 s$^{-1}$ and 88000 M$^{-1}$ s$^{-1}$ for WNV protease and 3.6 μM, 0.02 s$^{-1}$ and 5500 M$^{-1}$ s$^{-1}$ for 
DEN2 [29]. Data obtained for $k_{cat}/K_m$ with the substrate peptides 
Ac-nKRR-amc and Bz-nKRR-amc revealed a discernible contribution 
of the N-terminal protection group on the activity of the respective substrate, whereby the benzoyl moiety contributes to an 
approximately 1.4-fold higher $k_{cat}/K_m$ when compared to the 
acetyl group.

Although the internally quenched substrate Abz-(R)4SAGnY-
amide was shown previously to be efficiently cleaved by DEN 
NS2B(H)-NS3pro, ($k_{cat}/K_m$: 11087 M$^{-1}$ s$^{-1}$) [22], binding affinity 
and cleavage efficiency ($k_{cat}/K_m$: 19 M$^{-1}$ s$^{-1}$) of this peptide was 
substantially lower (approx. 500-fold) for JEV NS2B(H)-NS3 
protease than for the DEN NS3 protease. This finding is in 
agreement with notable differences in substrate preferences 
between JEV and DEN NS3 as assumed from a comparison of 
cleavage site sequences present in the viral polyprotein precursor 
[29].

The effect of pH on the enzymatic activity of JEV protease was 
determined by assays using buffers in the pH range from 6.5–11.0 
(Fig. 5, panel A). In agreement with earlier reports on flaviviral 
proteases [15,24,25,36] the pH optimum for reaction with the 
peptide substrate Ac-nKRR-amc was 9.5. It is noteworthy that the 
avtivity of the enzyme at physiological pH is less than 25% of the 
avtivity observed at pH 9.5.

Flaviviral serine proteases exhibit marked inhibition by high salt 
concentrations [24,25], and we examined effects of elevated ionic 
strength by high concentrations of NaCl (Fig. 5, panel B). 
Cleavage of the substrate Pyr-RTKR-amc was inhibited by about 
50% in the presence of 25 mM NaCl, which can be compared to 
the 40–50% inhibition of the dengue virus NS3 protease caused by 
100 mM NaCl, as earlier reported [24,37], thereby suggesting an 
even greater sensitivity of the JEV NS3 and MVEV proteases to 
high ionic strength.
Earlier studies have shown that the activities of flaviviral proteases from DEN and WNV are comparatively insensitive to inhibition by conventional protease inhibitors, like PMSF and benzamidine [25,47]. We here evaluated the inhibitory activities of aprotinin on the JEV protease. Dose-response plots in the presence of increasing inhibitor concentrations assayed with the Pyr-RTKR-amc substrate suggest IC$_{50}$ values of $4.13^{+0.17}_{-0.17}$ µM for aprotinin (Fig. 6). Previous studies have reported $K_{i}$ values for aprotinin of $0.16^{+0.02}_{-0.02}$ µM for WNV NS3 protease, and $0.02^{+0.01}_{-0.01}$ µM for DEN2 NS3 protease [15]. Similarly, for MVEV NS2B-NS3, the IC$_{50}$ of aprotinin is about 8 µM [14]. In another study, using a non-cleavable form of WNV NS2B-NS3pro, the $K_{i}$ of aprotinin was reported as 26 nM [38].

Thus, in comparison, JEV NS3 protease appears to be even less susceptible to this protease inhibitor evaluated here.

### 3.3 Structural Comparisons of JEV Protease with WNV, DEN and YFV Proteases

The alignment of the JEV, WNV DEN2 and YFV polyprotein sites cleaved by NS2-NS3 proteases is shown in Fig. 7. As seen, the JEV and WNV sites are the most similar; they all contain Lys-Arg at the P2-P1 positions, while at the P1 position a Gly is present at
four out of the five sites (the exception is the NS3/NS4A-junction, which contains a Ser). The DEN2 and YFV sites are less similar with those of JEV, however; in six cases out of ten, these sites have Arg-Arg at the P2-P1 positions, while the four remaining sites have different positively charged residues (Lys-Arg, Arg-Ser, Gln-Arg and Arg-Lys) at these positions. It is also notable that the five cleavage sites of JEV contain five different amino acids at P3; the same holds true for P4.

The multiple sequence alignment of the WNV, JEV, DEN2 and YFV NS2B-NS3 proteases is shown in Fig. 8. The alignment revealed that the JEV protease is closest to WNV, with a sequence identity of 76.3% (93.7% sequence similarity), while the DEN2 and YFV proteases show only 50.2% and 45.5% sequence identity (79.5% and 76.9% sequence similarity) with the JEV protease, respectively.

The X-ray crystal structure of the WNV protease/inhibitor complex NS2B(H)-NS3-protease-Bz-nKRR-H [27] indicates that the WNV NS2B-NS3 protease has four distinct substrate binding pockets, termed S4-S1, which accommodate the P4-P1 residues of protease substrates [39]. The S1 pocket is composed of nine residues; the key interactions with P1 Arg are being formed by the side chain of Asp129 and the backbone carbonyl oxygen of the Tyr130 residue of NS3. A large part of the S1 pocket comprises the aromatic side chains of Tyr150 and Tyr161 of NS3. In the X-ray structure Tyr161 is forming a cation-π stacking with P1 arginine, which is located between Tyr161 and Bz cap of the inhibitor; possible cation-π stacking of substrate P1 arginine with Tyr150 is also suggested from mutagenesis studies [40,48]. The S2 pocket of the WNV protease is outlined by eight residues and is dominated by a negative electrostatic potential from backbone carbonyl oxygen atoms of Asp82 and Gly83 of NS2B, and Val71 and Lys72 of NS3, as well as from acidic side chains of Asp82 of NS2B and the active site triad. S3 and S4 pockets are less well defined and consist of a few (three and two, respectively) uncharged amino acids, which makes the substrate P4 and P3 residues being largely solvent exposed. The sequence stretches contributing to the S4-S1 pockets are marked in the multiple alignment of Fig. 8. As seen, JEV and WNV share the same amino acid at 17 of the 22 indicated positions; JEV and DEN2 share the same amino acid at 11 positions and JEV, and YFV at 12 positions. However, there are positions in each one of S4-S1 pockets, which contain physico-chemically quite different amino acids. Residue 84 of NS2B contributes to the S2 pocket, and is Asp, Asn, Ser and Glu in, respectively, JEV, WNV, DEN2 and YFV. Residue 86 of NS2B is part of the S3 pocket and contains also four different amino acids in the four viruses (His, Gln, Ser,
Positions 132 and 155 of NS3, which belong to S1 and S4 pockets, respectively, are also different in the different proteases. These amino acid differences are thus likely candidates to contributing to the differences in kinetics of substrates for the different proteases.

In order to cast further light into this possibility, we built a homology model of the JEV NS2B-NS3 protease, using the crystal structure of a WNV NS2B(H)-NS3-protease-Bz-nKRR-H inhibitor complex as template. The model for the JEV protease is shown superimposed on the WNV protease/inhibitor complex in Fig. 9, panel A. Pairwise 3D structure alignment showed that the JEV and WNV proteases share large structural similarity (RMSD = 0.172 Å), with a large structural conservation in the enzymes’ active sites. However, clear differences are seen in the substrate-binding pockets at NS2B residues Asn84, Gln86, and NS3 residues Thr132, Ile155 (WNV NS2B-NS3 protease numbering, corresponding to NS2B residues Asp84, His86, and NS3 residues Arg132, Glu155 in the JEV protease). This is visualized in Fig. 9, panels B and C. Side chains of all four residues are in close proximity with the ligand and are capable of forming multiple hydrogen bonds (except Ile 155 in the WNV protease). The superimposition shows that the change of these residues between JEV and WNV leads to a serious rearrangement of the H-bond network in the substrate-binding pocket, which very likely will affect the cleavage preferences of the two proteases. Moreover, while all these residues are uncharged in the S1-S4 pockets of the WNV protease, the corresponding residues in the S2 and S4 pockets of the JEV protease are acidic (Asp and Glu) while in the S1 pocket it is basic (Arg). All of this may influence both the ionic interactions with substrates and the geometries of the binding pockets.

Discussion

Over the past decade, the NS2B-NS3 two-component serine proteases of human-pathogenic flaviviruses have received substantial scientific interest as potential drug targets, as these enzymes are indispensable components of virus replication

\[13,19,41\]. Compared to the NS3 proteases of the closely related DEN and WNV, the NS3 protein from JEV is much less well characterized. In this study we report a straightforward procedure for producing recombinant JEV NS2B(H)-NS3pro protease by overexpression in *E. coli* followed by one-step purification procedure, and we report the kinetic parameters of the protease for commercially available synthetic fluorogenic model peptide substrates and serine protease inhibitors.

The full-length NS2B-NS3 polypeptide region of JEV was obtained by time- and cost-efficient gene synthesis, and was used...
as template for the generation of the NS2B(H)-NS3pro protease complex comprising residues 51–95 and residues 121–131 from the NS2B cofactor and N-terminal residues 1–180 of the NS3 protease domain. Previous studies have demonstrated that such a construct is catalytically active both in proteolytic autocleavage conducive to the formation of a non-covalent NS2B(H)-NS3pro complex and with peptide substrates supplied for in trans cleavage reactions [24].

The recombinant NS2B(H)-NS3pro protein produced by *E. coli* was predominantly biosynthesized as a soluble protein, and could be easily purified by metal chelate affinity chromatography to 95% purity. In SDS-PAGE analysis, the purified protein displayed bands of 36, 21 and 10 kDa, corresponding to the NS2B(H)-NS3pro precursor, the NS3pro protease domain and the histidine-tagged NS2B(H) cofactor, respectively. Similar to the protein from DEN, the 29.8 kDa (His)6NS2B(H)-NS3pro protein of JEV exhibits anomalous migration in SDS-PAGE gels. In contrast to the corresponding protein from DEN serotype 2, the NS2B(H)-NS3pro protein from JEV was largely soluble upon expression in *E. coli*, thus suggesting the existence of major conformational differences between the two proteins.

In analogy to a number of specificity studies on the NS3 proteases from WNV, YFV, and JEV [15,29,42,43], we assayed a small set of commercially available fluorogenic peptide substrates, based on P3-P1 sequences of cleavage sites (capsid protein, NS2B/NS3 itself, NS3/NS4A, and NS4B/NS5 cleavage site) of NS2B/NS3 proteases of WNV, YFV, and JEV. In addition, we assayed an internally quenched peptide, Abz-(R)4SAGnY-amide, representing the P4-P3’ sequence of the capsid protein cleavage site of the DEN-2 virus [22].

Among all substrates used, the highest binding affinity and cleavage efficiency was observed for the Pyr-RTKR-amc substrate, which represents the native JEV NS2B/NS3 cleavage site at the P3 to P1 positions. The binding affinity for the substrate with a leucine at P3 (Boc-LRR-amc) was three-fold higher when compared to that with a glycine at P3 (Boc-GRR-amc); however, the catalytic efficiency for Boc-GRR-amc was about 10-fold higher than for Boc-LRR-amc, thereby suggesting a significant contribution of this position to the catalytic mechanism as seen in an earlier report for the DEN NS3 protease [44]. The LRR-amc substrate also displays the lowest catalytic efficiency of all tested peptides. Boc-GRR-amc and Boc-LRR-amc were previously assayed with the NS3 proteases from DEN and WNV, where it was found that both enzymes prefer a Gly residue to a Leu at the P3 position [15,44]. Our results indicate that hydrolysis of substrates with a short chain amino acid (Gly) at the P3 position is favoured by the JEV NS2B(H)-NS3 protease over those with a bulky residue (Leu), which aligns with previous data reported for the DEN2 and WNV proteases [15,45]. The prominent contribution to catalytic efficiency of residues at the P3 and P4 position (and possibly
prime-side residues) for the JEV protease is also reflected by the comparatively weak activity of the internally quenched peptide Abz-(R)4SAGnY-amide, which is an efficient NS3 substrate originally designed from the capsid protein sequence RRRR of DEN2 [22]; here it demonstrated a 580-fold lower $k_{\text{cat}}/K_m$ when assayed with the JEV enzyme, compared with DEN2. Whereas the tetrabasic non-prime side sequence is strongly favoured by DEN NS3, the presence of Asn and Gln at P3 and P4 of JEV apparently confers a high difference in specificity between the two enzymes. Moreover, it can not be ruled out that the prime-side sequences (SAG in DEN and GGN in JEV) contribute substantially to the decrease in efficiency as seen for this substrate peptide.

Although kinetic data for the JEV protease are relatively limited to date, it can be concluded that the enzyme from JEV favours substrates with Lys-Arg at the P2-P1 position, while the NS3 proteases from DEN and YFV prefer Arg-Arg at this position; data which thus suggest a greater similarity of the JEV protease to WNV than to DEN [15]. This view is supported by amino acid sequence alignments, the crystal structures for the enzymes from DEN and WNV, and structure-guided mutagenesis studies, which show that functional determinants of activation and substrate recognition for JEV and WNV are more closely related than other flaviviral proteases (Fig. 7). It is noteworthy that both the whole sequence and the substrate binding regions of the NS2B-NS3 protease JEV show much higher identity to WNV than to DEN2 and YFV proteases (Fig. 8 and [46]).

We carried out multiple sequence alignment of the NS2B-NS3 proteases of WNV, JEV, DEN and YFV, which allowed identifying differences in the putative binding pockets of the proteases (Fig. 8). We identified four amino acid residues (positions 84 and 86 in NS2B and 132 and 155 in NS3), which were different for all four proteases. In order to confirm the role of these residues in substrates binding and cleavage preferences of NS2B-NS3 proteases, we built a 3D homology model of the JEV NS2B-NS3 protease and compared its substrate binding pocket with that of the WNV NS2B-NS3 protease (Fig. 9). The structural modeling revealed that the conformation of the JEV protease is overall highly similar with the structure of the WNV NS2B-NS3 complex [21,27,45]. However, it can be seen that the change of Asn84, Gln86, Thr132 and Ile155 in WNV NS2B-NS3 protease to Asp84, His86, Arg132 and Glu155 in the JEV NS2B-NS3 protease leads to rearrangement of the possible H-bonds between the ligand and the S1, S3, and S4 pockets of the enzyme (Fig. 9, panel B, C). Moreover, presence of an acidic amino acid in S4 and a basic amino acid in S1 may influence both the binding pocket geometry and ligand-protease interactions. This makes the proteases of the two viruses quite different in terms of their binding mode, affinity and cleavage preferences and are likely reasons for the observed differences in the cleavage efficiency of substrates for the JEV and WNV enzymes.

An earlier study employing hybrid NS2B-NS3 constructs of DEN and JEV sequences showed that only a [DENNS2B-(JEV)NS3 protein could efficiently process the JEV polyprotein, whereas the [JEVNS2B-(DEN)NS3 construct was inactive, supporting the notion that NS2B proteins of different origins modulate the structure and substrate affinity of the protease [21,20]. This also indicates that there are likely differences in the
conformational space between the NS2B-NS3 proteases of different flaviviruses.

The protein inhibitor aprotinin was found to inhibit JEV NS2B/NS3 protease with surprisingly low potency (IC$_{50}$: 4.15±0.167 µM); this inhibitory efficiency is comparable with the previously reported inhibitory efficiency of aprotinin for the MVEV NS3 protease (IC$_{50}$: 7.8±2.9 µM) [14]. It is noteworthy that there are several reports where differences in the susceptibility of the WNV protease to aprotinin was seen [48,49], possibly suggesting a modulation of inhibitor sensitivity by recombinant sequences introduced by genetic engineering.

To sum up, in this study we present for the first time a comparative enzyme-kinetic analysis of a recombinant JEV protease by employing model substrate peptides. Our data demonstrate that the JEV protease shows marked differences to other known flaviviral proteases. Our study may serve as an entry point to the development of efficient JEV inhibitors, e.g. by employment of high-throughput screening.

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Author Contributions

Conceived and designed the experiments: GK JW CA. Performed the experiments: MJ CC AST. Analyzed the data: GK MJ JS JW. Contributed reagents/materials/analysis tools: GK CA. Wrote the paper: MJ JS ML GK JW.

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