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Biochemical characterisation of Murray Valley encephalitis virus proteinase

Joma Joy, Ng Fui Mee, Wee Liang Kuan, Kwek Zekui Perlyn, Then Siew Wen, Jeffrey Hill *

*Experimental Therapeutics Centre, Agency for Science, Technology and Research (A*Star), 31 Biopolis Way, #03-01 Nanos, Singapore 138669, Singapore*

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ABSTRACT

Murray Valley encephalitis virus (MVEV) is a member of the flavivirus group, a large family of single stranded RNA viruses, which cause serious disease in all regions of the world. Its genome encodes a large polyprotein which is processed by both host proteinases and a virally encoded serine proteinase, non-structural protein 3 (NS3). NS3, an essential viral enzyme, requires another virally encoded protein co-factor, NS2B, for proteolytic activity. The cloning, expression and biochemical characterisation of a stable MVEV NS2B–NS3 fusion protein is described.

1. Introduction

Murray Valley encephalitis virus (MVEV) is a member of the *Flaviviridae*, a large family of single stranded RNA viruses. Many members of the family are causative agents of serious disease in both humans and animals. The *Flaviviridae* is composed of three genera, *Flavivirus*, *Pestivirus* and *Hepacivirus*. The Flavivirus genus is the largest and contains over 70 viruses including MVEV, yellow fever virus (YFV), dengue virus (DENV), Japanese encephalitis virus (JEV) and West Nile virus (WNV) [1]. Each genus can be further subdivided into serocomplex, clade and cluster [2].

MVEV derived its name from a region of South Australia where in the 1950's one of the first recorded outbreaks of encephalitis caused by the virus occurred [3]. MVEV is related to, but distinct from JEV, both being members of the JE serocomplex. Like all flaviviruses, MVEV has a positive sense, single stranded RNA genome that encodes a single open reading frame which is translated into a polyprotein precursor [4]. The amino terminus of the polyprotein is composed of three structural proteins (capsid, envelope and membrane) and the remainder of the precursor consists of seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) that are involved in viral replication. Post-translational proteolysis of the polyprotein is an essential process required for viral replication and it is performed by host proteinases such as furin and the virally encoded proteinase NS3. NS3 is a multi-functional protein consisting of a nucleotide triphosphatase, an RNA triphosphatase, a helicase and a trypsin-like serine proteinase domain at the N-terminus [5]. The NS3 serine proteinase contains the archetypal catalytic triad (His-51, Asp-75, Ser-135) and recognises the highly specific cleavage sequence of two basic residues in P2 and P1, with a small unbranched amino acid in P1'. Different flavivirus proteinases show a preference for substrates with either Lys or Arg at P2 [6]. The proteinase activity of NS3 is dependant on an association with a 40 amino acid hydrophilic region of NS2B protein. Recombinant DENV2 virus proteinase has been generated where the N-terminal 184 residues of NS3 were covalently linked to the essential co-factor of NS2B by a Gly_4-Ser-Gly_4 linker [7] and this has enabled the characterisation of DENV2 viral proteinase and a host of other flaviviral proteinases including WNV proteinase [8], YFV proteinase and St. Louis encephalitis virus proteinase [9]. In addition to biochemical characterisation, the crystal structures of WNV proteinase, DENV2 proteinase [10] and the MVEV proteinase-helicase complex [5] have been solved. However, one problem that has been observed with this construct is a lack of stability due to autoproteolysis, the NS3 protease domain cleaving itself from the covalently linked NS2B cofactor. An optimisation study was performed on WNV proteinase where the autocatalytic cleavage site was identified and removed as well as unnecessary regions of the glycine linker and the cofactor domain [11]. The optimum NS3 proteinase size was also determined and it was shown that the optimised NS2B/NS3 protein had very similar substrate binding properties to full length NS3 proteinase.

Abbreviations: MVEV, Murray Valley encephalitis virus; YFV, yellow fever virus; JEV, Japanese encephalitis virus; WNV, West Nile virus; NS3, non-structural protein 3

* Corresponding author.
E-mail address: jhill@etc.a-star.edu.sg (J. Hill).

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This report describes the generation of an optimised recombinant MVEV NS2B/NS3 proteinase complex whose design is based on the optimisation experiments performed on the WNV proteinase. To the authors' knowledge, this is the first report of an investigation into the biochemical characteristics, the substrate preferences and the inhibitor profiles of the proteinase encoded in the MVEV genome.

2. Materials and methods

2.1. Generation and expression of MVEV proteinase

The protein sequences of NS2B and NS3 (Accession number: NP_051124) from MVEV were aligned with the corresponding sequences from WNV (Accession number: AAP22088). This allowed the identification of the homologous regions of the MVEV proteins that have been determined previously in WNV to be optimal for the generation of a recombinant proteinase molecule [11]. The two protein sequences were linked with a Gly_4-Ser-Gly_3 linker, a 6-His tag was added at the N-terminus and the resulting sequence, hereon referred to as $NS2BNS3_{MVEV}$, was reverse translated using UpGene, a web based DNA codon optimisation tool [12]. The reverse translation generated a nucleic acid sequence that was optimised for expression in an *Escherichia coli* host. A synthetic gene was synthesised (GenScript, Piscataway, NJ, USA) with *Nco*I and *Sal*I sites at the 5′ and 3′ ends of the gene to allow in-frame subcloning into the expression vector pET21d(+) (Merck, Darmstadt, Germany).

Gene expression in BL21(DE3) cells (Merck) was performed as previously described [13]. The recombinant proteinase was purified by immobilised affinity chromatography (IMAC) and then desalted on the Profinia Protein Purification System (Bio-Rad, Hercules, CA, USA). The purity of the enzyme was assessed by SDS–PAGE and by analysis on the 2100 Bioanalyzer (Agilent, Foster City, CA, USA). An accurate size determination of the protein was performed by mass spectrometry using the Agilent 6530 quadrupole time of flight liquid chromatography–mass spectrometer.

2.2. Biochemical and kinetic characterisation of recombinant MVEV proteinase

The activity of the recombinant proteinase complex was established by demonstrating the cleavage of the flourogenic peptide Pyr-Arg-Thr-Lys-Arg-AMC (Bachem, Bubendorf, Switzerland), an optimised furin substrate [14]. The assay was performed in a 96 well plate in a final reaction volume of 0.1 ml using essentially the same conditions as previously described (20 nM enzyme, 10 mM Tris–HCl, pH 8.0, 1 mM CHAPS, 30% glycerol) [8]. The enzyme was pre-incubated in reaction buffer for 10 min at 37 °C before the reaction was initiated by the addition of the peptide substrate to a final concentration of 30 μM. The components of the reaction were mixed with a 5 s shake and the progress of the reaction was monitored continuously at 37 °C by measuring the increase in fluorescence (λ_{ex} 355 nm and λ_{em} 460 nm) every 43 s for 30 min. on a SpectraMax Gemini XS plate reader. Relative activity was calculated and expressed in terms of relative fluorescent units generated per minute (RFU/min.). This assay procedure was used to determine the optimum pH for enzyme activity under defined reaction conditions. The range of pH conditions were established by using different buffering systems, pH 6.0 (10 mM MES), pH 7.0 (10 mM MOPS), pH 8.0 and 9.0 (10 mM Tris–HCl), pH 10.0 (10 mM CAPS) and pH 11.0 (10 mM piperidine). The ionic strength of each of the buffers used was calculated using the Proteomics and Functional Genomics, University of Liverpool, web-based tool (www.liv.ac.uk/buffers). The ionic strength of all the buffers used was in the 3–4 mM range. Flaviviral proteinases have previously been shown to be inhibited by elevated concentrations of NaCl [15], so to determine if this was the case for $NS2B/NS3_{MVEV}$ proteinase, a series of assays were performed to measure enzymatic activity as NaCl concentration was increased (0–200 mM). It has been reported that glycerol is an essential component of all flaviviral proteinase assays [16] so assays were performed in the presence of increasing glycerol concentrations (0–75%). All of the biochemical assays were performed in triplicate and mean values calculated.

The kinetic parameters for $NS2B/NS3_{MVEV}$ proteinase were determined for the aforementioned furin substrate and for an optimised DENV2 proteinase substrate (Bz-Nle-Lys-Arg-Arg-AMC) [17]. The assays were performed using nine different substrate concentrations (0–100 μM) in triplicate. Reaction velocities were measured, curve fitting was performed using GraphPad Prism 5, by selecting the Michaelis Menten equation and K_m values were determined. Kinetic parameters were also determined for the inhibition of the $NS2B/NS3_{MVEV}$ proteinase by the archetypal serine proteinase inhibitor aprotinin and a nona- D-Arg-NH_2 peptide. The enzyme was pre-incubated with varying concentrations of inhibitor at 25 °C for 60 min. Following addition of substrate, the reaction was monitored as described above. Dixon plots ($1/v$ versus [I]) were plotted using GraphPad Prism 5 and IC_{50} values were derived. K_i values were calculated using the equation $K_i = IC_{50}/(1 + S/K_m)$. All assays were performed in triplicate.

3. Results and discussion

Expression of the codon-optimised construct encoding the $MVEV_{NS2BNS3}$ proteinase complex resulted in approximately 100 mg of recombinant protein per litre of culture, all of which was soluble. A single step affinity purification followed by desalting was sufficient to generate protein of over 92% purity, as assessed on the Bioanalyzer, at a final yield of 16 mg of purified protein per litre of culture. The size of the protein appeared to be in excess of 30 kDa when analysed by SDS–PAGE and a value of 34 kDa was assigned by the Bioanalyzer. There is some discrepancy between the theoretical value of 26352.45 Da and the electrophoresis derived values and this anomaly has been observed before with other recombinant flaviviral proteinases (personal communication). Analysis by mass spectrometry however, determined the mass to be 26221.71 Da. It has been reported that N-terminal methionine residues are readily removed from recombinant proteins produced in *E. coli* by the action of methionine aminopeptidase (MAP) and more readily if the adjacent residue is a non-bulky one [18]. The residue adjacent to the initiating methionine in the proteinase construct expressed was an alanine. Therefore, it is not unreasonable to conclude that the methionine residue of the recombinant $MVEV_{NS2BNS3}$ was cleaved off by MAP. The theoretical mass of the recombinant $MVEV_{NS2BNS3}$ with the N-terminal methionine removed is 26221.45 Da, less than 0.001% different from the mass spectrometry derived value, further supporting the supposition of MAP activity.

Cleavage of the flourogenic furin substrate demonstrated the proteolytic activity of the recombinant proteinase and allowed the characterisation of the enzyme. The reaction buffer used contained 1 mM CHAPS as previous studies have shown a non-ionic detergent to enhance activity, although little difference between detergents used was reported [16]. The pH optimum of $MVEV_{NS2BNS3}$ was found to be pH10.0 with a rapid decline as pH increased (Fig. 1a). To ensure that the AMC containing flourogenic peptide substrate was stable at the range of pH values investigated, it was incubated for 30 min in the appropriate reaction buffer. Fluorescence was measured at 0 and 30 min, no significant decrease in fluorescence was observed at any pH. The concentration of NaCl was very influential on proteinase activity, with activity reduced by more than 60% at concentrations exceeding 25 mM (Fig. 1b). This finding is consistent with studies performed on other

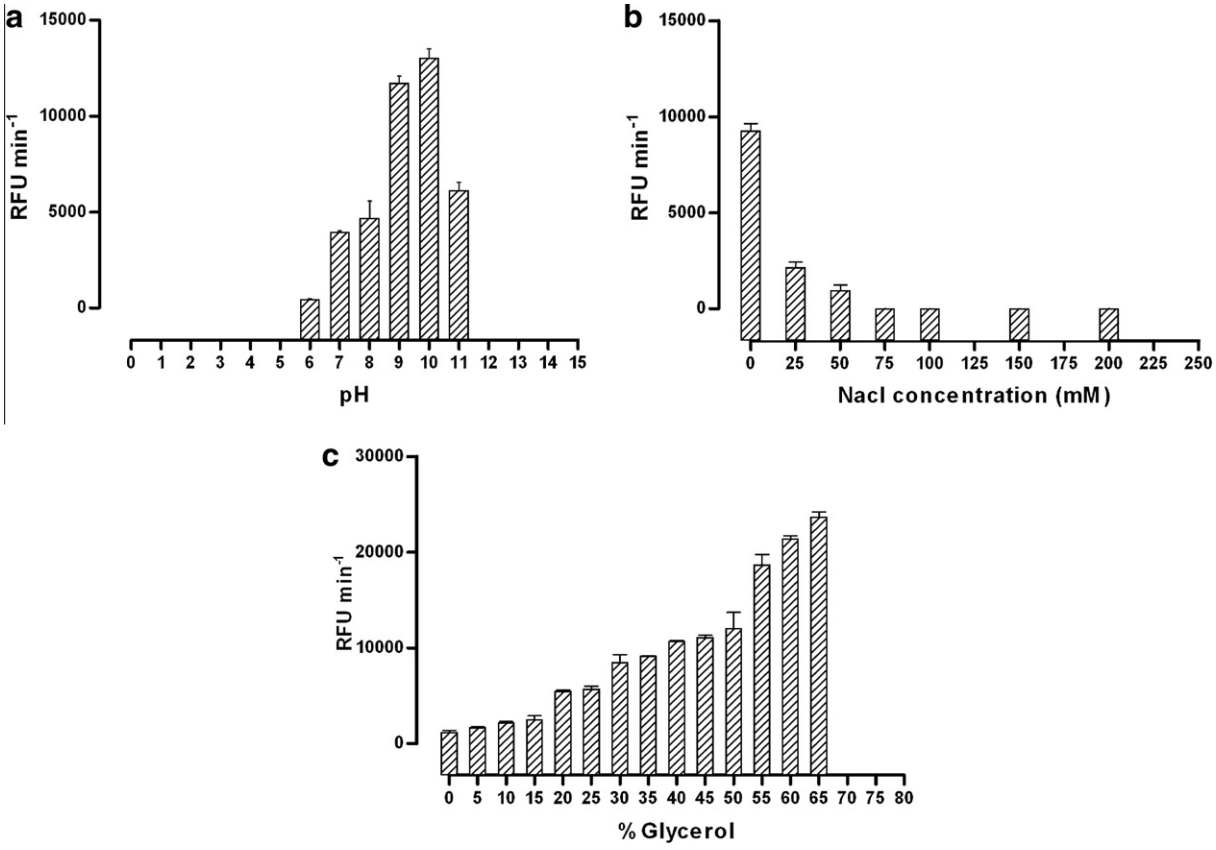

Fig. 1. The effect of pH, glycerol concentration and ionic strength on the proteolytic activity of $MVEV_{NS2BNS3}$ was carried out in the presence of 30 μM of substrate (Pyr-RTKR-AMC). The assay buffer contained 20 nM enzyme, 30% glycerol, and 1.0 mM CHAPS. (a) The effect of pH on the protease activity was determined over a range of pH (6.0–11.0) whilst maintaining almost constant ionic strength (I = 3.0–4.0 mM). The buffers used were 10 mM MES (pH 6.0), 10 mM MOPS (pH 7.0), 10 mM Tris (pH 8.0, 9.0), 10 mM CAPS (pH 10.0) and 10 mM piperidine (pH 11.0). (b) The ionic strength dependence of the MVEV protease assay was determined by varying concentration of NaCl (0–200 mM). (c) The effect of glycerol was assayed by varying the glycerol concentration (0–65%) in the assay buffer.

flaviviral proteinases [19]. To further investigate the effect of NaCl on proteolytic activity, K_m and K_{cat} values were derived using the standard assay buffer at various concentrations of NaCl. At 1 mM NaCl the K_m value more than doubled with a 30% reduction in K_{cat} demonstrating the negative impact of ionic strength on substrate binding (data not shown). In fact, reliable kinetic parameters could not be derived at NaCl concentrations above 1 mM. Glycerol also had a profound effect on proteinase activity, relative activity being approximately proportional to glycerol concentration up to a final concentration of 65% (Fig. 1c). However, handling of solutions with such high concentrations of glycerol is problematic, so a compromise of maximal activity versus ease of use was adopted, using 30% glycerol in the subsequent proteinase assays. The design of the $MVEV_{NS2BNS3}$ proteinase construct was based on the optimisation work of Chappell et al. to improve the stability of recombinant WNV proteinase [11]. In order to see if the same sequence selection criteria applied to the proteinase from MVEV, the recombinant $MVEV_{NS2BNS3}$ proteinase complex was incubated at 37 °C for 40 h. Analysis by SDS–PAGE revealed no auto-cleavage events up to 24 h of incubation and activity measurements revealed very little loss of relative activity (data not shown).

Kinetic parameters were derived for the interactions between $MVEV_{NS2BNS3}$ proteinase and the furin and DENV2 proteinase substrates. The binding affinity of the enzyme for the DENV2 proteinase substrate was higher than for the furin substrate and was reflected in lower K_m values. It has been reported that residue 84 in the NS2B cofactor interacts with the P2 residue in the substrate and is a major determinant of binding affinity [6]. The proteinases from the four DENV serotypes have either a threonine or serine in this position and bind substrates with an arginine in P2 more tightly. Analysis of the natural cleavage sites of the polyprotein from the four DENV serotypes almost always shows an arginine in the P2 position. Whereas the proteinase from WNV has an asparagine in position 84 and has higher affinities for substrates with a lysine in P2. The P2 position in the WNV polyprotein is always lysine. MVEV, like all members of the JE serotype, has an aspartic acid residue in position 84 of NS2B and a lysine residue in the P2 sites of the polyprotein, so the tighter binding of substrates with an arginine in the P2 position is of note (Table 1).

Polyarginine containing peptides have been shown to be potent inhibitors of furin, the mammalian subtilisin/Kex2p like proteinase [20]. The enzyme has a similar substrate preference for a dibasic

Table 1
Kinetic parameters for the hydrolysis of the two synthetic substrates, Pyr-Arg-Thr-Lys-Arg-AMC and Bz-Nle-Lys-Arg-Arg-AMC by $NS2B/NS3_{MVEV}$ proteinase.

Substrate	K_m (μM)	V_{max} (pmol min^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Pyr-RTKR-AMC	47.89 ± 3.88	18.03 ± 0.627	0.150 ± 0.005	0.00313×10^6 ± 0.0003
Bz-Nle-KRR-AMC	26.53 ± 3.15	33.24 ± 1.79	0.277 ± 0.029	0.0104×10^6 ± 0.0003

Table 2
Kinetic parameters for the inhibition of NS2B/$NS3_{MVEV}$ proteinase by aprotinin and a nona-D-Arg-NH_2 peptide.

Inhibitor	IC_{50} (μm)	K_i (μm)
Nona-D-Arg-NH_2	0.072 ± 0.007	0.037 ± 0.004
Aprotinin	7.8 ± 2.9	4.1 ± 1.5

sequence in P1P2 positions as do the flaviviral proteinases. Therefore it is not surprising that the viral and mammalian enzymes share common inhibition profiles. Indeed, it has been reported that D-arginine based peptides, that are potent inhibitors of furin, potently inhibit WNV proteinase [21]. A nona D-arginine peptide was found to inhibit NS2B/$NS3_{MVEV}$ proteinase with an IC_{50} of approximately 72 nM and a K_i of 37 nM (Table 2), compared to the K_i value reported for WNV proteinase of 6 nM.

The protein inhibitor aprotinin was found to inhibit NS2B/$NS3_{MVEV}$ proteinase with surprisingly low potency in the low micromolar range ($IC_{50} \sim 8$ μM). However, reports of inhibition of other flaviviral proteinases by this inhibitor are varied. For example, one group reported that aprotinin inhibits WNV proteinase with an IC_{50} of approximately 1 μM [22], yet another group reported an IC_{50} of approximately 100 nM [23]. The IC_{50} of WNV proteinase inhibition by aprotinin determined in this study was 1 μM (data not shown). One possible explanation for this discrepancy may be the use of different recombinant proteinase constructs. The work of Robin et al. [22], which reported an IC_{50} value in the low micromolar range, uses the same construct design described by Chappell et al. [11] as does this report. The work of Johnston et al. [23], which reported an IC_{50} in the low nanomolar range, uses a construct that differs in the linker region between the NS2B cofactor and the NS3 protease domain by utilising a Gly_4-Ser-Gly_4 sequence in contrast to the optimised Gly_3-Ser-Gly_4 linker sequence. Also the lysine residue at position 48 of NS2B, adjacent to the glycine linker, was mutated to an alanine residue to prevent autocatalytic cleavage. It appears that the two different strategies to increase the stability of the recombinant proteinase have differentially affected the binding properties of the enzyme to the protein inhibitor aprotinin.

MVEV is endemic in Northern Australia and Papua New Guinea where there are a small number of cases each year with a fatality rate of approximately 20% [24]. There are no vaccine development programs underway at present due to the low number of cases but it is possible that vaccines being developed to combat JEV may be efficacious against MVEV. There are also no anti-viral drug discovery programs ongoing for the same economic reasons. However, there are substantial resources being invested in the discovery of inhibitors of key viral enzymes, including the proteinase, from a number of other flaviviruses including WNV and DENV1-4. A pan flaviviral proteinase inhibitor developed primarily for use against dengue fever or WNV encephalitis may be appropriate for use in other flaviviral infections including MVEV encephalitis. To enable this to take place a detailed characterisation of MVEV proteinase is required. Although a crystal structure has recently been solved for the proteinase–helicase complex [5], this report describes the first study to generate a stable proteinase moiety and characterise its proteolytic activity.

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