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Protein Expression and Purification 52 (2007) 334–339
Protein Expression & Purification
www.elsevier.com/locate/yprep

Expression and purification of a two-component flaviviral proteinase resistant to autocleavage at the NS2B–NS3 junction region

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Received 1 September 2006, and in revised form 6 November 2006
Available online 1 December 2006

Abstract

Regulated proteolysis of the polyprotein precursor of West Nile virus (WNV) by the essential NS2B–NS3(pro)tease, a promising drug target for WNV inhibitors, is required for the propagation of infectious virions. Structural and drug design studies, however, require pilot-scale quantities of a pure and catalytically active WNV protease that is resistant to self-proteolysis. Autolytic cleavage at the NS2B–NS3 boundary leads to individual, non-covalently associated, NS2B and NS3 domains, together with residual amounts of the intact NS2B–NS3, in the NS2B–NS3pro samples. We modified the cleavage site sequence of the NS2B–NS3 junction region and then developed expression and purification procedures to prepare a covalently linked, single-chain, NS2B–NS3pro K48A mutant construct. This construct exhibits high stability and functional activity and is thus well suited for the follow-up purification and structural and drug design studies.

Keywords: West Nile virus; Flaviviruses; NS2B–NS3; Proteolysis; Polyprotein processing; Mutagenesis; Autoproteolysis

Introduction

West Nile virus (WNV)[1], a member of the Flaviviridae family and an emerging pathogen in the US, is transmitted to animals, including humans, by mosquito bites [1,2]. There are no specific countermeasures against WNV infection in humans. The WNV capsid encloses a single-stranded RNA encoding a 3400 amino acid residue polypeptide precursor. The precursor is comprised of three structural proteins [C, capsid, M, membrane, and E, envelope] and seven non-structural (NS) proteins arranged in the order C–prM–E–NS1–NS2A–NS2B–NS3–NS4A–NS4B–NS5 [3,4]. Proteases from the host (furin and secretase) and from the virus [NS3 serine proteinase (NS3pro)] are required to process the polyprotein precursor into individual functional proteins [5,6]. NS3pro is responsible for the cleavage of the capsid protein C and at the NS2A/NS2B, NS2B/NS3, NS3/NS4A, NS4A/NS4B and NS4B/NS5 boundaries [7–14].

The full-length NS3 peptide sequence is a multifunctional protein. The N-terminal 184 amino acid residue fragment represents the serine proteinase NS3pro with a conventional His–Asp–Ser catalytic triad. The C-terminal portion of the NS3 protein encodes an RNA helicase. During virus propagation, protease and helicase normally work together in a coordinated fashion [15]. As is the case with a number of flaviviruses, the NS2B protein that is located in the polypeptide precursor upstream of the NS3pro domain functions as a cofactor and promotes the folding and the functional activity of the NS3pro [16–18]. The individual NS3pro domain, lacking the NS2B part, is catalytically inert. The cofactor activity of the 35–48-residue central portion is approximately equivalent to that of the entire NS2B sequence [19,20]. Inactivating mutations of the NS3pro cleavage sites in the polyprotein precursor abolish viral

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[1] *Abbreviations used:* WNV, West Nile virus; C, capsid; M, membrane; E, envelop; DV, dengue virus.

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doi:10.1016/j.pep.2006.11.009

infectivity [3]. These data suggest that the two-component NS2B–NS3pro is a promising drug target for WNV inhibitors. The sequence of WNV NS2B–NS3pro is 56% identical to that of Dengue virus (DV), thus suggesting a high degree of structural similarity that exists between these two proteinases. A crystal structure of the individual DV NS3pro complexed with mung-bean Bowman–Birk type trypsin inhibitor has been available for several years [21,22]. This structure, however, represents a virtually inactive protease. When our work was in progress, the high resolution structures of the two-component WNV and DV NS2B–NS3pro in complex with the substrate-based inhibitor benzoyl-norleucine (P4)-lysine (P3)-arginine (P2)-arginine (P1)-aldehyde (Bz–Nle–Lys–Arg–Arg–H) became available [17].

Structural and drug design studies require the availability of substantial amounts of recombinant NS2B–NS3pro with high proteolytic activity and resistance to self-proteolysis. Normally, because of self-proteolysis of the NS2B–NS3 junction region, individual, non-covalently associated NS2B and NS3 domains, together with residual amounts of intact NS2B–NS3, are present in the NS2B–NS3pro samples [18]. Their presence complicates the isolation and analysis of NS2B–NS3pro. To overcome this difficulty, we modified the cleavage site sequence of the NS2B–NS3 junction region in order to obtain covalently linked, single-chain NS2B–NS3pro that exhibits high stability and functional activity, well suited for follow-up purification and structural and drug design studies.

Materials and methods

Reagents

Reagents were purchased from Sigma–Aldrich (Milwaukee, WI) unless indicated otherwise. Pyroglutamic acid-RTKR-7-amino-4-methylcoumarin (Pyr–RTKR–AMC) was purchased from American Peptide (Sunnyvale, CA).

Enzyme cloning, expression and purification

Cloning of the DNA sequence encoding the wild-type two-component NS2B–NS3pro from WNV was described earlier [18]. The 48 amino acid residue central portion of NS2B (residues 1393–1440 of the WNV polyprotein precursor) and the NS3 (residues 1476–1687 of the WNV polyprotein precursor) sequences were linked by a flexible GGGGSGGGG linker. The WNV autolytic site-deficient NS2B–NS3pro(K48A) construct was prepared with 5′-CCAGGAGCACCTTGGGCGGGCGGGGGAGGT-3′ and 5′-ACCTCCCCCGCCCGCCCAAGGTGCTCCTGG-3′ forward and reverse primers, respectively (mutant nucleotides are underlined) using a QuickChange mutagenesis kit (Stratagene, San Diego, CA). After confirming their authenticity by sequencing, the constructs were re-cloned into the pET101 expression vector. Competent *Escherichia coli* BL21 (DE3) Codon Plus cells (Stratagene, San Diego, CA) were transformed with the recombinant pET101 vectors. Transformed cells were grown at 30 °C in a Luria–Bertani broth containing ampicillin (0.1 mg/ml). Cultures were induced with 0.6 mM isopropyl β-D-thiogalactoside for 16 h at 18 °C. *Escherichia coli* cells (6 g/L of *E. coli* culture) were then collected by centrifugation (5000*g*; 15 min), resuspended in 20 ml PBS containing 1 M NaCl, 1 mM phenylmethylsulfonyl fluoride and lyzozyme (5 mg/ml), and disrupted by sonication (30 s pulse, 30 s interval; 8 pulses) on ice. The debris were then removed by centrifugation at 20,000*g*, 30 min.

The NS2B–NS3pro WT and K48A constructs, C-terminally tagged with a 6 × His tag, were each purified from the soluble fraction of *E. coli* cell extract using affinity chromatography on a 1.6 × 10 cm Co^{2+}-chelating Sepharose Fast Flow column (Amersham, Piscataway, NJ) equilibrated with PBS–1 M NaCl–1 mM phenylmethylsulfonyl fluoride. NS2B–NS3pro was eluted with 100 ml of 10–500 mM gradient of imidazole in PBS–1 M NaCl–1 mM phenylmethylsulfonyl fluoride buffer (elution rate 0.5 ml/min). The fractions (1.5 ml each; 10 fractions total) containing NS2B–NS3pro were collected and diluted 10-fold with PBS and the protease samples were then re-chromatographed under the same conditions on a Co^{2+}-chelating Sepharose Fast Flow column. The presence of NS2B–NS3pro in the fractions was confirmed by 4–20% gradient gel electrophoresis and also by the activity assay. In the activity assays 1 μl fraction aliquots and a Pyr–RTKR–AMC substrate were used. The NS2B–NS3pro K48A protein fractions were concentrated with a 5 kDa-cutoff concentrator (Millipore, Billerica, MA) and dialyzed against 10 mM Tris–HCl, pH 8.0, containing 0.005% Brij 35. Typical yields of the purified NS2B–NS3pro K48A were 10 mg/L of *E. coli* culture.

Protease assays with fluorogenic peptides

The assay for NS3pro cleavage activity was performed in 10 mM Tris–HCl buffer, pH 8.0, containing 20% glycerol and 0.005% Brij 35. The Pyr–RTKR–AMC substrate and enzyme concentrations, unless indicated otherwise, were 24 M and 10 nM, respectively. The total assay volume was 0.1 ml. Initial reaction velocities were monitored continuously at $_{ex}$360 nm and $_{em}$460 nm on a Spectramax Gemini EM fluorescence spectrophotometer (Molecular Devices, Sunnyvale, CA). All assays were performed in triplicate in a 96 well plate. The values of K_m and k_{cat} were derived from a double reciprocal plot of $1/V_0$ vs. $1/[S]$, using the Lineweaver–Burk transformation: $1/V_0 = K_m/V_{max}\ x\ 1/[S] + 1/V_{max}$, where V_0 is the initial velocity of the substrate hydrolysis and $[S]$ is the substrate concentration, V_{max} is the maximum rate of hydrolysis, and K_m is the Michaelis–Menten constant. The concentration of the catalytically active proteinase was measured using a fluorescence assay by titration against a standard aprotinin solution of a known concentration.

Circular dichroism spectroscopy of the NS2B–NS3pro constructs

CD spectra (190–250 nm) of the NS2B–NS3pro and NS2B–NS3pro K48A purified samples (both at 1.4 mg/ml in