Supplementary information, Data S1 Materials and Methods

Protein cloning, expression, and purification

The cDNA of Zika virus (ZIKV), encoding NS2B residues 48-92 and NS3 residues 1-170 connected by a G_4SG_4 linker, was subcloned in between the *Bam*HI and *Hind*III sites of pET-duet-1 vector (Novagene). The NS2B-NS3pro was expressed in Rosetta™ (DE3) strain. The cell cultures were grown in Luria–Bertani broth containing 100 mg/l ampicillin and 20 mg/l chloramphenicol at 37 °C. Protein expression was induced with 0.5 mM isopropyl β-D-thiogalactoside for 16-20 h at 16 °C. The recombinant protein was purified by a nickel column, followed by an anion exchange column (HiTrap Q HP) and a HiLoad 16/600 Superdex 75 pg column (GE Healthcare). The Δloop-S2 (deletion of residues 79-92 from NS2B) and Δloop-S1 (deletion of residues 159-170 from NS3pro) mutants were expressed and purified by a similar method.

Enzymatic activity and inhibition assays

The enzymatic activity of NS2B-NS3pro and the inhibition of aprotinin were measured by continuous kinetic assays, using a fluorogenic substrate AC-LKRR-AMC (>95% purity, GL Biochem Shanghai Ltd., Shanghai, China). The fluorescent intensity was monitored with a Fluoroskan Ascent instrument (Thermo Scientific, USA) using wavelengths of 355 and 460 nm for excitation and emission, respectively. The assay was performed in a buffer solution consisting of 10 mM Tris-HCl (pH 9.0), 20% glycerol, and 1 mM CHAPS at 37 °C. The kinetic parameters (K_m and k_{cat}) were determined with measurement of initial rates. The reaction was initiated by adding substrate to the reaction buffer system containing 0.1 μM

protease. The range of final substrate concentrations was 3.125-150 μM. The fluorescence was monitored per 10 s. Initial rates were calculated by fitting the linear portion of the curves using GraphPad Prism 5. Kinetic constants were determined using double-reciprocal plot based on Michaelis-Menten method.

Inhibition assay was performed in a 100 μl buffer system containing 10 mM Tris-HCl (pH 9.0), 20% glycerol, 1 mM CHAPS, 0.1 μM enzyme, 100 μM substrate, and varying concentrations of inhibitors. Typically, the NS2B-NS3pro (at a final concentration of 0.1 μM) was pre-incubated with various concentrations of inhibitors at 37 °C for 10 min. The reaction was then initiated by adding the substrate to a final concentration of 100 μM. IC_{50} was determined using Dixon plots (v_i/v_0 versus Log[I]). K_i was calculated from IC_{50} based on the equation, $K_i = IC_{50}/ (1+[S]/K_m)$. Triplicate measurements were performed for each data point. The final result was presented as mean ± S.E.

Crystallization, data collection and structure determination

The ZIKV NS2B-NS3pro was concentrated to 30 mg/ml and then subjected to crystallization screening using microbatch-under-oil method at 293 K. Crystals with a typical size of 0.1×0.1×0.1 mm were obtained in 0.1 M Bis-Tris pH 6.5, 0.05 M calcium chloride dihydrate, 30% v/v polyethylene glycol monomethyl ether 550 in 12 days. Crystals were cryo-protected by using Parabar 10312 (previously known as Paratone oil) before flash-frozen in liquid nitrogen. A complete data set diffracted to 2.6 Å resolution was collected on beamline BL18U at Shanghai Synchrotron Radiation Facility (SSRF) using a Pilatus3 6M detector at a wavelength of 0.97853 Å. The intensity data were indexed, integrated and scaled with the

HKL-2000 package[1]. The crystal belonged to the space group $P4_12_12$ with two NS2B-NS3pro molecules in the asymmetric unit. The structure of NS2B-NS3pro was determined by molecular replacement using the program PHASER[2] implemented in the CCP4 package[3]. The structure of West Nile virus NS2B-NS3 protease (PDB ID: 2GGV) was used as the search model. Iterative rounds of model rebuilding in COOT[4] and restrained refinement in REFMAC5[5] led to a final model with R_{work} of 22.4% and R_{free} of 27.3%. The statistics for diffraction data and structure refinement are summarized in Supplementary information, Table S1.

Supplementary References:

1 Otwinowski Z, Minor W. Processing of X-ray Diffraction Data Collected in Oscillation Mode. In: Carter CW, Jr. , Sweet RM. eds. Macromolecular Crystallography, part A. New York, NY: Academic Press 1997:307-326.

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