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Effects of Detergents on the West Nile virus Protease Activity

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Abstract

Detergents such as Triton X-100 are often used in drug discovery research to weed out small molecule promiscuous and non-specific inhibitors which act by aggregation in solution and undesirable precipitation in aqueous assay buffers. We evaluated the effects of commonly used detergents, Triton X-100, Tween-20, Nonidet-40 (NP-40), Brij-35, and CHAPS, on the enzymatic activity of West Nile virus (WNV) protease. Unexpectedly, Triton X-100, Tween-20, and NP-40 showed an enhancement of in vitro WNV protease activity from 2 to 2.5-fold depending on the detergent and its concentration. On the other hand, Brij-35, at $\geq 0.001\%$ enhanced the protease activity by 1.5-fold and CHAPS had the least enhancing effect. The kinetic analysis showed that the increase in protease activity by Triton X-100 was dose-dependent. Furthermore, at Triton X-100 and Tween-20 concentrations higher than 0.001%, the inhibition of compound B, one of the lead compounds against WNV protease identified in a high throughput screen (IC_{50} value of $5.7 \pm 2.5 \mu M$), was reversed. However, in the presence of CHAPS, compound B still showed good inhibition of WNV protease. Our results, taken together, indicate that nonionic detergents, Triton X-100, Tween, and NP-40 are unsuitable for the purpose of discrimination of true versus promiscuous inhibitors of WNV protease in high throughput assays.

Introduction

The family of *Flaviviridae* contains more than 70 viruses including Yellow fever virus (YFV), Dengue virus (subtypes 1-4) (DENV1-4), West Nile virus (WNV), Kunjin, and Japanese encephalitis virus primarily transmitted by arthropods¹. The mosquito-borne flaviviruses such as Dengue and West Nile viruses have recently emerged in major epidemics causing severe and lethal diseases such as dengue hemorrhagic fever and WNV encephalitis with significant morbidity 2-3. Currently, there is no antiviral therapy or vaccines available for treatment or prevention of DENV and WNV infections.

Flaviviruses are composed of a positive-sense RNA which is translated into a single polyprotein comprising three structural (C, prM, E) and seven non-structural (NS) proteins arranged in the order NH_2 -C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH. The polyprotein is processed by the host proteases in the endoplasmic reticulum at the C-prM, prM-E, and E-NS1

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as well as NS4A-NS4B sites and by the viral serine protease at specific sites containing two basic amino acid residues at P1 and P2 positions of the substrate followed by Ala, Ser, or Gly at the P1' position (according to the nomenclature of Schechter and Berger) within the nonstructural regions¹.

The NS3 protein was identified as a multifunctional protein exhibiting enzymatic activities of a serine protease within the N-terminal region as well as an NTPase, 5'-RNA triphosphatase, and RNA helicase within the C-terminal region⁴⁻¹². The serine protease domain (NS3-pro; ~184 aa) consists of three highly conserved amino acids, His51, Asp75, and Ser135, in the catalytic triad¹³⁻¹⁴. In previous studies, it was shown that NS3-pro requires the NS2B (130 aa) cofactor for protease activity in processing of the polyprotein at NS2A-NS2B, NS2B-NS3, NS3-NS4A, and NS4B-NS5 sites¹⁵⁻²¹. NS2B consists of three hydrophobic regions flanking a conserved ~40 residue hydrophilic domain (NS2BH) which is sufficient for protease activity *in vitro*^{20-22,23}. The role of flavivirus protease in polyprotein processing, which is a prerequisite for viral RNA replication, renders the protease as an attractive target for development of inhibitors as potential therapeutics.

One of the frequently used experimental approaches in identification of small molecule lead compounds is high throughput screening (HTS) based on robust *in vitro* as well as cell-based assays. Using the HTS, hundreds of thousands of compounds can be screened within a short time. However, potential false-positive "hits" could arise due to compounds having undesirable properties such as aggregation leading to nonspecific inhibition. Compounds which exhibit these properties are known as "promiscuous" inhibitors, which could inhibit a variety of targets in a nonspecific manner^{24,25}. The aggregates of compounds may envelop the protein and block the binding site for the substrate inhibiting the protein's function²⁴⁻²⁶. To identify such compounds and eliminate them from the list of true inhibitors, previous studies have reported the use of detergents that modulate surface properties and cause disaggregation of the compounds²⁴⁻²⁷. Thus, if a compound shows inhibition in the presence of a detergent, it is then considered as a true lead compound that warrants further analysis. Triton X-100 is commonly used for distinguishing "false positive hits" from the true inhibitors²⁵. In this study, we demonstrate that Triton X-100 and Tween-20, and NP-40, the three nonionic detergents are not suitable for discrimination of true from "promiscuous" inhibitors in HTS because they enhance the WNV protease activity by 2- to 2.5-fold. However, a zwitterionic detergent, CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate), has no significant effect on protease activity *per se* and hence could be used to distinguish false positive hits from true inhibitors. A lead inhibitor of WNV protease previously identified by HTS shows good inhibition in the presence of CHAPS but not in the presence of Triton X-100.

Methods and Materials

Materials

Triton X-100 was purchased from ICN Biomedicals. The purified WNV protease (NS2B(H)/NS3-pro) was obtained as reported previously²⁸. The fluorogenic WNV substrate, *t*-butyl-oxycarbonyl (Boc)-Gly-Lys-Arg-7-amino-4-methylcoumarin (AMC), was purchased from Bachern. Compound B was purchased from I.F. Lab (vendor ID, F0842-0004) (Life Chemicals Inc., Burlington, ON, Canada). CHAPS was purchased from Pierce, Rockford, IL.

In vitro protease assay

All assays were done in triplicate in black 96-well plates. The *in vitro* protease assays were performed as described before²⁸⁻²⁹. The reaction mixture of 100 μ l/assay contained 200 mM Tris pH 9.5, 30% glycerol, 27 nM WNV protease, 2% DMSO, and fluorogenic WNV substrate Boc-Gly-Lys-Arg-AMC at concentrations that varied from 15.5 μ M to 2 mM or at 100 μ M for

fixed substrate concentrations. In some assays, the effect of Triton X-100 or CHAPS on the WNV protease activity was verified using a tetrapeptide substrate, N-Carbobenzyloxy-Val-Lys-Lys-Arg-4-Methoxy- β -naphthylamide. Aprotinin (bovine pancreatic trypsin inhibitor, BPTI) was used as a positive control for inhibition of WNV protease ($K_i \sim 162$ nM)²⁸ at a final concentration of 10 μ M, and dimethylsulfoxide (DMSO) for the no-inhibitor control at a concentration of 2%. In vitro protease assays in presence of Triton X-100 were performed by adding the detergent to the protease buffer (31 nM WNV protease/200 mM Tris-HCl, pH 9.0/30% glycerol). The fluorescence of the AMC, the product of the protease activity on the tripeptide substrate was measured at excitation and emission wavelengths of 385 and 465 nm, respectively. The hydrolysis of the tetrapeptide substrate to the product, 4-methoxy-b-naphthylamine, was monitored at excitation and emission wavelengths of 290 and 420 nm, respectively. Fluorescence was measured using the SpectraMax Gemini EM spectrofluorometer (Molecular Devices). The relative fluorescence units (RFU) were converted to micromolar (μ M) concentrations of AMC using a standard curve generated from the fluorescence values versus micromolar concentrations of free AMC. Kinetic values were determined using Microsoft Excel and GraphPad Prism 5 (La Jolla, CA). RFUs, obtained in a standard assay in the absence of detergents, were taken as 100% (control).

To assess the effects of inhibitors in the presence and absence of detergents, protease assays were performed as above except in the presence of various concentrations of an inhibitor (in the range, 0.3125-100 μ M), serially diluted in dimethylsulfoxide (DMSO; final concentration, 2%). In these assays, the inhibitor-enzyme complex was allowed to form by preincubation for 15 min at room temperature before addition of the substrate (100 μ M final concentration). After five minutes incubation, fluorescence was measured as described above. From the initial velocities of the reactions, the IC_{50} values were determined using GraphPad Prism 5. The lowest and highest fluorescence values were set as 100% and 0% inhibition, respectively.

Effect of Triton X-100 on kinetic constants of WNV protease

In the standard assay, the WNV substrate concentrations were varied from 0 to 2 mM to determine the kinetic parameters, K_m and V_{max} , of the WNV protease in the presence of DMSO (2%). Time course of WNV protease reaction, monitored by Spectra Max Gemini EM, were analyzed by linear regression to obtain the slopes in RFU/min. This value was then converted to μ M/min using a standard curve of free AMC as described above. Using GraphPad Prism 5 software, initial rates (v) of enzyme activity were fit by non-linear regression analysis into

Michaelis-Menten equation $v = \frac{V_{max} \times [S]}{[S] + K_m}$ where the V_{max} and K_m are the maximum velocity and Michaelis-Menten constant, respectively, and $[S]$ is the substrate concentration.

Results

(i) Triton X-100 enhances the WNV protease activity in a dose-dependent manner

Since Triton X-100 is often used to discriminate between the “false positives” hits and the true inhibitors in the in vitro assays, we first tested the effect of Triton X-100 on the activity of WNV protease. The non-ionic detergents, Tween-20, NP-40, and Brij-35 as well as the zwitterionic detergent, CHAPS, were also used for comparison. As shown in Fig. 1, Triton X-100, Tween-20, and NP-40 enhanced the relative activity of the protease at concentrations $\geq 0.001\%$. The level of enhancement reached about 2- to 2.3-fold when these detergents were present in the range of 0.01 -0.1%. This stimulatory effect of Triton X-100 is not unique to the tripeptide substrate (Boc-Gly-Lys-Arg-AMC) used in the protease assay. The same enhancement effect of Triton X-100 was also seen when the tetrapeptide substrate, 4-methoxy-naphthylamide, was used (Fig. 1C). Another non-ionic detergent, Brij-35, enhanced the protease activity only about 1.5-fold in the same range of detergent concentration. On the other

hand, the protease activity was not significantly affected by the zwitterionic detergent, CHAPS (Fig. 1).

(ii) Effect of Triton X-100 on the K_m and the V_{max} of the WNV protease

The K_m and V_{max} values were determined for the WNV protease in presence and absence of Triton X-100 to examine whether the detergent affects the affinity of the enzyme for the substrate. As shown in Fig. 2, the K_m values do not differ significantly in the presence and absence of Triton X-100 whereas the V_{max} values are increased with increasing concentrations of Triton X-100 (Table 1) in a dose-dependent manner. This experiment was repeated three times with similar values for K_m and V_{max} supporting our conclusion.

(iii) Effect of Triton X-100 on the inhibition of WNV protease by compound B

Compound B was identified as a lead inhibitor against WNV protease with a K_i of $3.4 \mu\text{M} \pm 0.6 \mu\text{M}$ in the in vitro assay and selectivity index of 100 in a replicon-based assay in mammalian (Vero) cells²⁹. Therefore, we sought to examine the inhibitory effect of the lead compound in the presence of Triton X-100. As shown in Fig. 3, when Triton X-100 was included at concentrations well below its critical micelle concentration (up to 0.001%) (CMC= ~0.02%), compound B still retained its inhibitory activity. However, at 0.01% Triton X-100 (~0.5 CMC) in the assay, the percent inhibition of the WNV protease activity by compound B was essentially abolished (Fig. 3A and 3B). However, the inhibitory activity of bovine pancreatic trypsin inhibitor (BPTI), also known as aprotinin, which is a potent inhibitor of DENV and WNV proteases with K_i values of 26 and 162 nM, respectively²⁸, was reduced only by about 20% in the presence of Triton X-100 (Fig. 1C and Fig. 3A).

Discussion

Triton X-100, polyethylglycol tert-octylphenyl ether, is a nonionic detergent, and is used extensively for solubilization of membrane proteins and their biochemical characterization. In drug discovery field, this detergent is used to discriminate the “promiscuous” inhibitors from true inhibitors of a target protein or enzyme in the in vitro assays^{24,25,30,31}. Compounds that inhibit unrelated enzyme targets in the in vitro assays often do so by aggregation and/or formation of colloidal particles which could bind to the targets nonspecifically and interfere with substrate or inhibitor binding leading to false “hits”. The presence of a detergent below its CMC, provided it does not interfere with the assay, prevents aggregation of the inhibitor compound and allows interaction between the inhibitor and the target.

However, the findings of the present study using the WNV protease as the enzyme target indicate that Triton X-100 interferes with the inhibitory activity of compound B (Fig. 3) and other compounds tested (data not shown). However, inclusion of a zwitterionic detergent such as CHAPS did not reverse the inhibitory activity of compound B (Fig. 1A). There are other examples of Triton X-100, Nonidet P-40 (NP-40) and/or Tween-20 interfering with target assays and those that describe that CHAPS or Brij-35 could also be used as a detergent to distinguish between true inhibitors from false-positive ones^{32-34,35}. For example, it was previously reported that Triton X-100 may act as a nonspecific activator of chymotrypsin³⁶. In the investigation of small molecule inhibitors of Rce1 p protease, which is required for Ras GTPase maturation, Triton X-100, NP-40, and CHAPS at their CMC inactivated the protease. Tween-20 at its CMC, on the other hand, was also ineffective for this purpose. Therefore, these detergents could not be used for distinguishing promiscuous versus true inhibition by the compounds³⁷. Moreover, Triton X-100 and NP-40 decreased the inhibition of reverse transcriptase (RT) activity by inophyllum B whereas CHAPS had no effect³⁸. In this regard, it was noted that Triton X-100 is an activator of HIV-1 RT activity and reverses the inhibition of TIBO R82150³⁹.

The possibility that compound B is a promiscuous inhibitor rather than a specific inhibitor of WNV protease is unlikely because of the following properties of compound B as an inhibitor of WNV protease. (1) Compound B shows some specificity as it did not have any inhibitory effect on cellular serine proteases such as trypsin and factor Xa²⁹. (2) Other derivatives of compound B also exhibit potent inhibition of WNV protease activity in vitro (P. Viswanathan et al. unpublished results; data not shown). (3) Compound B inhibited WNV RNA replication in a cell-based assay with an EC₅₀ value of $1.4 \pm 0.4 \mu\text{M}$ ²⁹. Moreover, in the presence of Triton X-100 at 0.001% compound B showed good inhibition of WNV protease with no change in the IC₅₀ value compared to no-detergent control (Fig. 3B). In the presence of Triton X-100 greater than 0.001% and Tween-20, the inhibition of WNV protease by compound B is reduced by up to 70% whereas the ionic detergent, CHAPS, did not have any effect. This study is the first to report on the enhancement of trypsin-like WNV serine protease activity by Triton X-100, Tween-20, and NP-40 whereas CHAPS shows no significant effect on the activity. In fact, the lack of appreciable stimulation by CHAPS up to 0.5% (CMC = 0.48% or 7.4 mM)⁴⁰ is not due to high pH of the assay buffer because it has been reported that CHAPS maintains its zwitter ionic property in a wide pH range of 2 to 12⁴¹. CHAPS was a component of the purification/assay buffer used in the characterization of the WNV protease activity in an earlier study²³; however, its property in the context of enhancing effects of detergents on the WNV protease activity was not investigated earlier to this study.

The mechanism by which Triton X-100 enhances the WNV protease activity is unknown at present. However, our kinetic analysis suggests that the detergent increases the V_{max} values of the enzyme in a dose-dependent manner without affecting the affinity of the enzyme (K_{m} values) for the substrate. One possibility is that Triton X-100, Tween-20, and NP-40 prevent the protease sticking to the wall of the 96-well plate thereby increasing the local concentration of the enzyme compared to the well without the detergent. Alternately, Triton X-100 may prevent aggregation of the enzyme thereby increasing the concentration of the active enzyme which would only influence the V_{max} but not the K_{m} . This possibility is supported by our data that K_{m} values for WNV protease remain almost the same, whereas V_{max} slightly increases with higher Triton X-100 concentrations (Table 1). However, the result that increasing the Triton X-100 concentration from 0.001 to 0.01 reduced the inhibition of the compound B by 70% (Fig. 3B), is not consistent with this interpretation because the total amount of the enzyme in each assay was only 2.7 pmol, the substrate 10 nmol and the inhibitor 2 nmol and therefore, increasing the concentration of the detergent is not expected to have any effect on the inhibition of compound B. Moreover, Triton X-100 does not have any effect on the substrate in the absence of WNV protease (data not shown). Another possibility should be considered that Triton X-100 at higher concentrations not only enhances the V_{max} of the hydrolysis of the substrate without significant change of K_{m} , but may also cause a conformational change at a different site that affects the binding of the compound B to the enzyme thereby attenuating its inhibition.

Therefore, our study emphasizes the need to test the effect of the chosen detergent on the enzyme (or any molecular target) prior to using it in the HTS for identification and elimination of false positive hits.

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Abbreviations

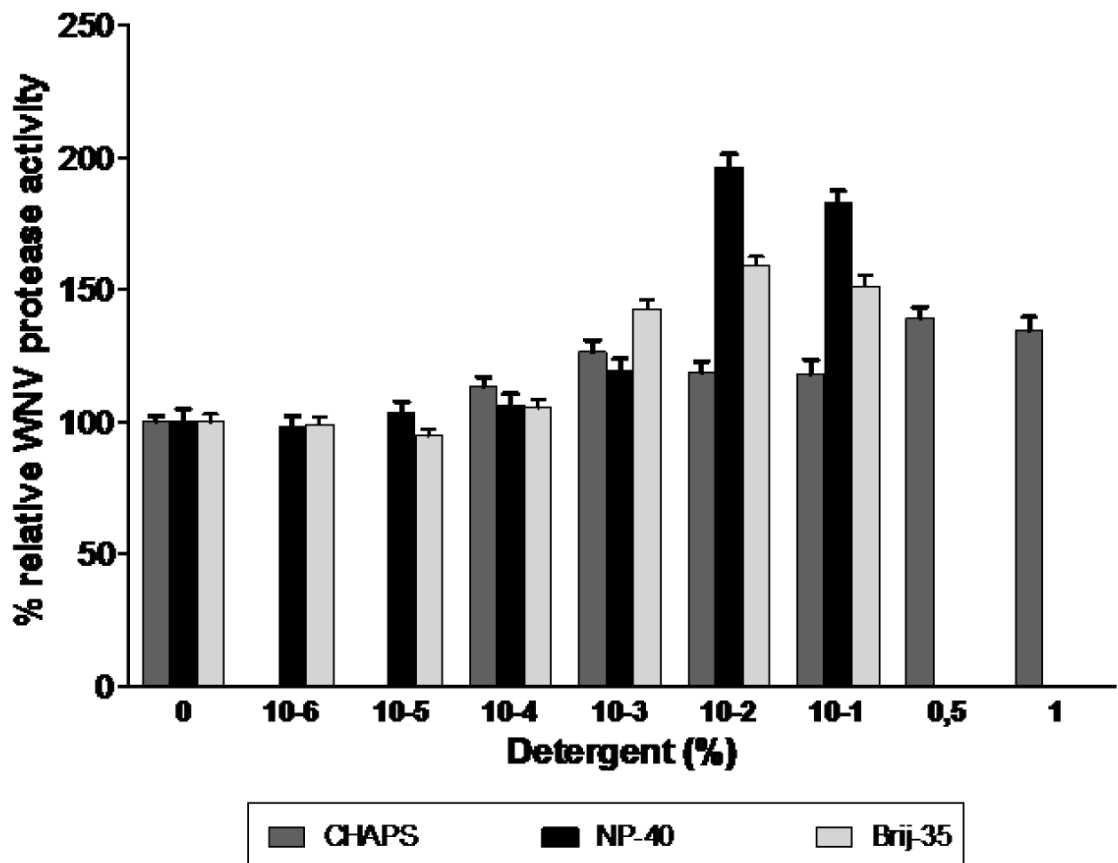
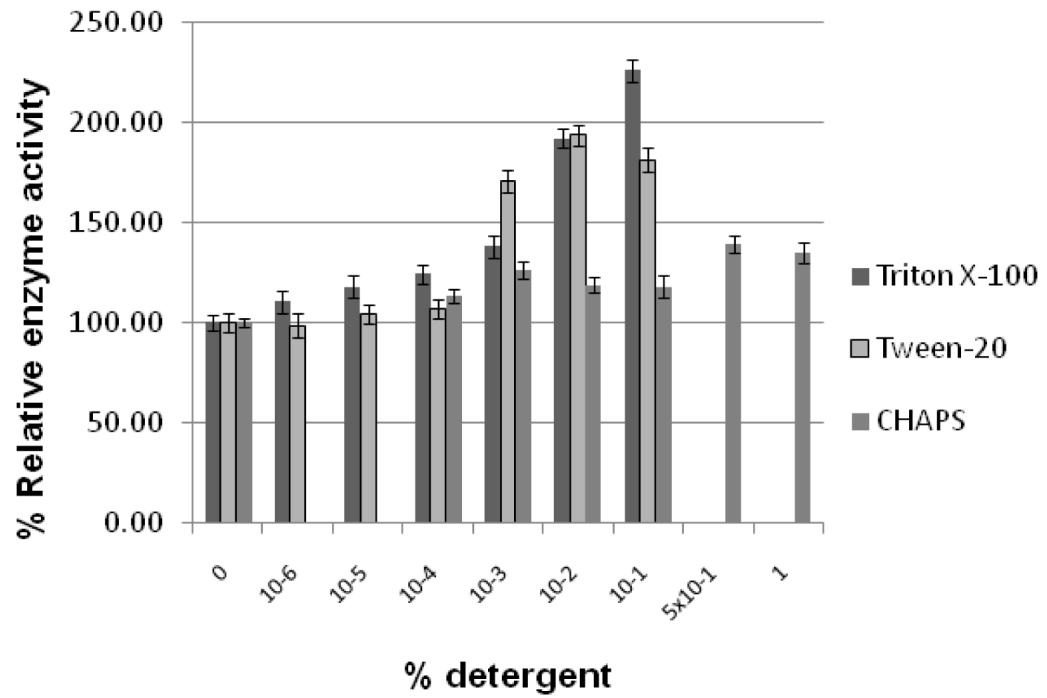
AMC 7-amino-4-methylcoumarin

CHAPS	(3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate)
CMC	critical micelle concentration
DENV	Dengue virus
DMSO	dimethylsulfoxide
IC ₅₀	50% inhibitory concentration
K _m	Michaelis-Menten constant
NS2BH	hydrophilic domain of non-structural protein 2B
NS3-pro	protein domain of non-structural protein 3
RFU	relative fluorescence unit
v _{max}	maximum enzyme velocity
WNV	West Nile virus

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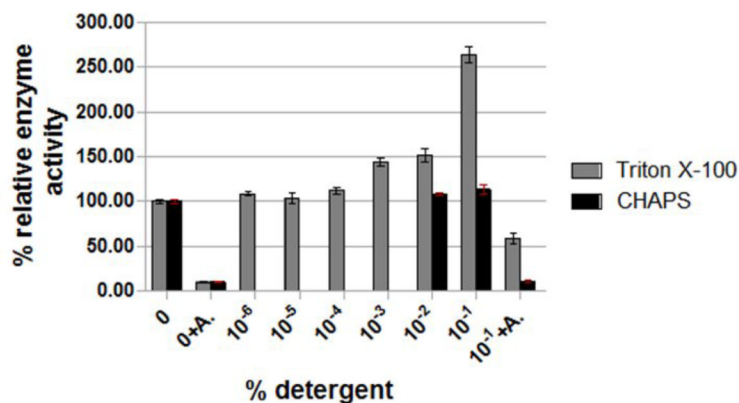


Figure 1. WNV protease activity in presence of different detergents

Panel A. Standard protease assays were carried out using the tripeptide substrate, *t*-butyl-oxycarbonyl (Boc)-Gly-Lys-Arg-7-amino-4-methylcoumarin (AMC) as described under Materials and Methods in the absence of any detergent or in the presence of Triton-X100, Tween-20, or CHAPS at the indicated concentrations of 10^{-6} , 10^{-5} , 10^{-4} , 10^{-3} , 10^{-2} , 10^{-1} , or 1 % in the assay mixture.

Panel B. The experimental conditions for the assay were the same as in Panel A except that NP-40, Brij-35, or CHAPS was used at the indicated concentrations.

Panel C. The conditions of the assay were the same except that the effects of Triton X-100 and CHAPS at indicated concentrations on the WNV protease activity using a tetrapeptide substrate, N-Carbobenzyloxy-Val-Lys-Lys-Arg-4-Methoxy-b-naphthylamide as described under Materials and Methods. The percent protease activity was plotted compared to that of the no-detergent control set at 100%.

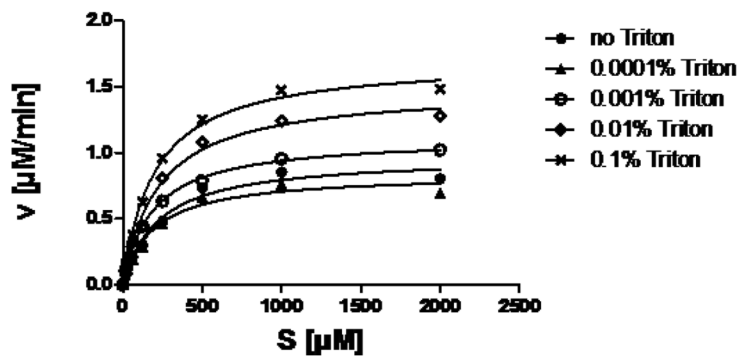


Figure 2. Effect of Triton X-100 on K_m and V_{max} values of WNV protease
Different WNV substrate concentrations (0 – 2000 μM) were used to determine WNV protease activity in the presence and absence of Triton X-100 in standard protease assays as described under Materials and Methods. The experiments were repeated three times and the error bars represent the standard deviation of the mean.

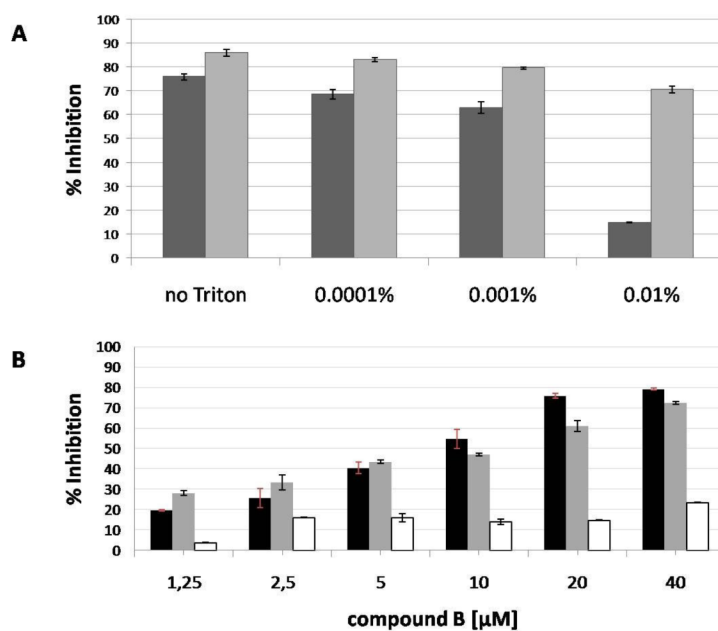


Figure 3. Effect of Triton X-100 on the inhibition of WNV protease by compound B

A. The assays were performed as described under Materials and Methods in the presence and absence of Triton X-100 and 2 μM aprotinin (light grey bars) or 20 μM compound B (dark grey bars). At Triton X-100 concentrations at 0.5 CMC (CMC=0.02%), the inhibition of compound B is reduced ~85%. **B.** The concentrations of compound B were varied as indicated. The protease assays were performed at two different Triton X-100 concentrations (0.001 %-grey bars and 0.01%-white bars) along with no-detergent control (black bars). The experiments in panels A and B were repeated at least three times with similar results. The error bars represent standard deviation of the mean.

Table 1Effect of Triton X-100 on the K_m and v_{max} values of WNV protease

Triton X-100 conc (%)	K_m [μM]	V_{max} [$\mu M/min$]	k_{cat} * [sec^{-1}]	k_{cat}/K_m 10^{-3} [$\mu M^{-1} sec^{-1}$]
0	172 \pm 60	0.97 \pm 0.18	0.60 \pm 0.11	3.5 \pm 0.6
0.0001	182.6 \pm 45	0.84 \pm 0.11	0.52 \pm 0.07	2.8 \pm 0.4
0.001	187.1 \pm 15	1.25 \pm 0.25	0.77 \pm 0.15	4.1 \pm 0.5
0.01	240 \pm 30	1.65 \pm 0.20	1.02 \pm 0.12	4.3 \pm 0.1
0.1	220 \pm 20	1.70 \pm 0.01	1.05 \pm 0.01	4.8 \pm 0.4

The protease assays were performed as described under Materials and Methods in the presence of indicated concentrations of Triton X-100. The K_m and V_{max} values were calculated using the GraphPad Prism 5.0 software.

* assuming a total enzyme concentration of 27 nM