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Yellow fever virus NS2B/NS3 protease: Hydrolytic Properties and Substrate Specificity

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ABSTRACT

Here we report the hydrolytic behavior of recombinant YFV NS2B/NS3 protease against FRET substrates mimicking the prime and non-prime region of the natural polyprotein cleavage sites. While the P2-P′1 motif is the main factor associated with the catalytic efficiency of Dengue (DV) and West Nile Virus (WNV) protease, we show that the k_{cat}/K_m of YFV NS2B/NS3 varied by more than two orders of magnitude, despite the presence of the same motif in all natural substrates. The catalytic significance of this homogeneity – a unique feature among worldwide prominent flavivirus – was kinetically analyzed using FRET peptides containing all possible combinations of two and three basic amino acids in tandem, and Arg and Lys residues produced distinct effects on k_{cat}/K_m. The parallel of our data with those obtained *in vivo* by Chambers et al. (1991) restrains the idea that these sites co-evolved with the NS2B/NS3 protease to promote highly efficient hydrolysis and supports the notion that secondary substrate interaction distant from cleavage sites are the main factor associated with the different hydrolytic rates on YFV NS2B-NS3pro natural substrates.

1. Introduction

Yellow fever (YF), a mosquito-borne flavivirus disease, is the prototypical viral hemorrhagic fever, characterized by a severe liver injury. According to the World Health Organization, there are 200,000 estimated cases of yellow fever, causing 30,000 deaths only in tropical South America and sub-Saharan Africa [1]. Despite the existence of a vaccine as an important prevention method, both the continued occurrence of wild-type YF and the incidence of vaccine-associated disease underlie the need of a clearer understanding of the YF pathogenesis and development of therapeutic interventions [2]. Moreover, no specific treatment for YF or any of the flaviviral infections is currently approved for use [3].

The family Flaviviridae comprises arthropod-borne enveloped virus with single- positive-stranded RNA genome like Yellow Fever virus (YFV), West Nile virus (WNV), Japanese Encephalitis Virus and Dengue virus (DV) [4,5]. The mRNA-like genomes of flavivirus are translated as large precursor polyproteins that are processed co- and post-translationally by host and viral proteases [6,7].These processing events are essential for flaviviruses replication and the viral protease is pointed out as an attractive target for antiviral development [8,9]. To achieve this goal, a detailed analysis of the substrate recognition specificity and catalytic properties of each flaviviral proteases are necessary.

Active flavivirus proteases are constituted by a two-domain protein formed by the association of a trypsin-like serine protease domain located at the N-terminal one-third of the viral non-structural protein 3 (NS3) with a hydrophilic region of 40 amino acids (CF40) of the NS2B protein [10]. NS2B domain acts as a cofactor that also actively participates in the formation of the enzyme active site [11,12].

The NS2B/NS3 protease catalyzes the cleavage in the non-structural region of viral polyproteins at the NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 sites, and at additional sites within the viral proteins C, NS4A, and within NS3 itself [13,14]. However, the amino acid sequence between the polyprotein processing sites of DV, WNV and YFV have similar but distinct composition. In DV, hydrolysis occurs on C-terminal side after a pair of basic residues (Lys-Arg, Arg-Arg, or Arg-Lys) or, occasionally, after Gln-Arg at the P_2 and P_1 positions (according to Schechter and Berger's nomenclature of substrate residues) followed by small amino acids (Gly, Ala or Ser) at P'_1 [4,15]. In the case of WNV, all the sites have Lys-Arg-Gly sequence (P_2-P'_1) with exception of the Lys-Lys-Gly motif found in NS2A internal cleavage site [16]. Interestingly, YFV NS3 polyprotein processing sites are restricted to the pair Arg-Arg followed by a small residue (Ser, Gly or Val) [4,17].

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While limited biochemical studies were performed with YFV NS2B/NS3 protease [7,18], the substrate specificity of DV NS2B/NS3 protease was assessed by different strategies and substantial information were obtained [19–23]. Above all, the use of positional scanning tetrapeptide libraries [23] and FRET substrates containing two or three basic amino acids in tandem [20] have defined the sequence (K)RR↓X (x = Ser, Gly or Ala) as optimal for DV NS2B/NS3 cleavage. However, while the Arg-Arg pair is associated with an increased DV NS2B/NS3 catalytic efficiency in FRET peptides based on polyprotein processing sites, interactions up to S_4 and S'_6 significantly influenced both k_{cat} and K_m values [20].

The unique presence of the Arg-Arg pair in YFV processing sites as well as its remarkable pathogenesis among flaviviruses, prompt us to analyze if these sites co-evolved with the NS2B/NS3 protease to promote highly efficient hydrolysis. Accordingly, we report here the hydrolytic activities of YFV NS2B/NS3 protease on seven (FRET) peptides based on native viral polyprotein processing sites and against two series of small FRET peptides containing all possible combinations of three and two basic amino acids in tandem arrangement flanked by small side chain amino acids.

2. Materials and methods

2.1. Enzymes

Recombinant YFV NS2B/NS3 protease was obtained and purified as previously described by [22]. Briefly, the 47 core amino acids of NS2B were linked (via Gly4-Ser-Gly4) to the N-terminally 190 amino acids of NS3 protease domain [18] and expressed in *E. coli* M15 strain as an N-terminally his-tagged fusion protein.

2.2. Peptide synthesis

All the FRET peptides were obtained by the solid-phase peptide synthesis strategy as previously described [24]. Stock solutions of peptides were prepared in DMSO, and the concentration measured spectrophotometrically using the molar extinction coefficient of 17.300 $M^{-1}cm^{-1}$ at 365 nm.

2.3. Hydrolysis of FRET peptides

The hydrolysis of FRET peptides were quantified using a Hitachi F–2500 spectrofluorimeter by measuring the fluorescence at 420 nm following excitation at 320 nm. The inner-filter effect was corrected as previously described [25]. The concentration of DMSO in assay buffers was kept below 1%. The scissile bond of hydrolyzed peptides were identified by isolation of the fragments using analytical HPLC followed by determination of their molecular mass by LC/MS using an LCMS-2010 equipped with an ESI-probe (Shimadzu, Japan).

2.4. Kinetic parameter determination

The kinetic parameters of hydrolysis, k_{cat}, K_m, and k_{cat}/K_m were determined from initial rate measurements at 8–10 substrate concentrations between 0.15 and 10 K_m. The enzyme concentrations were chosen such that less than 5% of the substrate was hydrolyzed over the course of the assay. The reaction rate was converted into micromoles of substrate hydrolyzed per minute based on a calibration curve obtained from the complete hydrolysis of each peptide. The data was fitted with respective standard errors to the Michaelis–Menten equation using GraFit software (Erithacus Software, Horley, Surrey, UK). In all assays data were collected at least in duplicate, and the error values were less than 10% for each of the obtained kinetic parameters.

2.5. The pH and salt dependence of specificity constant

The pH dependence of rate constants was measured under Michaelis–Menten conditions at 37 °C in a four-component buffer comprised of 75 mM Tris, 25 mM Mes, 25 mM acetic acid and 25 mM glycine, using the fluorimetric assay described above. The data were fitted to the theoretical curve for the bell-shaped pH rate profiles using nonlinear regression as in Eq. (1) using Grafit software:

$$k = \frac{k(\mathrm{Limit})10^{\mathrm{pH}-\mathrm{p}K_{a1}}}{10^{2\mathrm{pH}-\mathrm{p}K_{a1}-\mathrm{p}K_{a2}}} \quad (1)$$

Where k_{cat}/K_m(limit) stands for the pH-independent maximum k_{cat}/K_m constant and K_1 and K_2 are the dissociation constants of the catalytic components at acidic and basic limbs, respectively. $k = k_{cat}$ or k_{cat}/K_m. The pK_1 and pK_2 estimated from the pH–k_{cat}/K_m curves were identified as pK_{e1} and pK_{e2} respectively, to differentiate them from the pK_1 and pK_2 values estimated from the pH–k_{cat} profiles (pK_{es1} and pK_{es2} respectively). The influences of NaCl was investigated in 50 mM Tris, pH 9.0, using Abz-AKRRSQ-EDDnp as substrate.

2.6. Circular Dichroism

CD spectra were recorded on a Jasco J-810 spectropolarimeter with a Peltier system for controlling cell temperature. The absorbance spectra of FRET peptides were collected in the far-UV range (190–260 nm) using a 1 cm path length cell in standard buffer (Tris buffer (pH 9.0), 20% Glycerol). The system was routinely calibrated with an aqueous solution of twice crystallized d-10 camphorsulfonic acid. Ellipticity was recorded as the mean residue molar ellipticity [θ] (deg cm^2 $dmol^{-1}$). The spectrometer conditions typically included a sensitivity of 100 mdeg, a resolution of 0.5 nm, a response time of 4 s, a scan rate of 20 nm/min and 4 accumulations at 25 °C. The control baseline was obtained with the buffer prepared.

3. Results and discussion

3.1. Effects of pH and salt on the protease activity

The pH stability of YFV NS2B/NS3 protease was determined from the activity remaining at the optimal pH (9) following a 30-min pre-incubation at pH values of 7–10.5 at 37 °C. The enzyme retained more than 90% of its initial protease activity in the pre-incubated samples, thus indicating that the enzyme is stable in this pH range under our assay conditions (data not shown).

The pH dependence of the reaction of YFV NS2B/NS3 with the Abz-AKRRSQ-EDDnp substrate was measured under Michaelis–Menten conditions in the pH range from pH 7–10.5 at a constant-ionic strength buffer (25 mM MES, 25 mM acetic acid, 25 mM glycine, and 75 mM Tris). The substrate employed for this experiment (Abz-AKRRSQ-EDDnp) derived from the optimum sequence described for DV NS2B/NS3 protease [20]. As reported in Fig. 1A the k_{cat}/K_m values conform to "bell-shaped" pH-rate profile, in which the value of k_{cat}/K_m decreased at acid and basic pH, similar to those previously reported for other flaviviruses NS2B/NS3 proteases [7,13,20]. From the obtained data, for binding and catalysis of Abz-AKRRSQ-EDDnp titrated groups E_1 and E_2 have p$Ke1$ and p$Ke2$ values of 8.6 ± 0.1 and 9.9 ± 0.1, respectively, whereas optimal pH for proteolytic cleavage was found to be at pH 9.2.

The dependence of the catalytic constant k_{cat} with respect to pH was also examined (Fig. 1B). Bell-shape pH-profile obtained was broader than the k_{cat}/K_m profile due to a significative lowering of

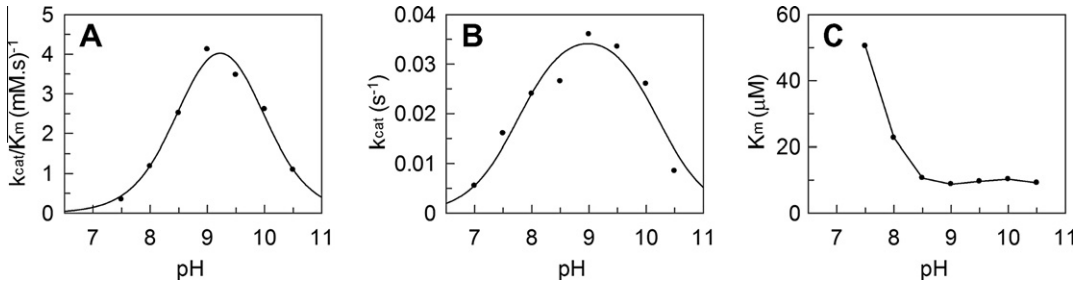

Fig. 1. pH dependency profile for YFV NS2B/NS3pro activity using Abz-AKRRSQ-EDDnp. Parameters of hydrolysis k_{cat}, K_m and the relationship k_{cat}/K_m were obtained for each pH under Michaelis–Menten conditions as described in Materials and Methods.

pK_{esl} and a small elevation of pK_{es2} upon substrate binding (pK_{esl} = 7.7 ± 0.2 and pK_{es2} = 10.2 ± 0.2). This profile is in agreement with the notion that formation of enzyme-substrate complex changes the accessibility to solvent of E_1 and E_2 groups.

Interestingly, when the pH dependence of the K_m value was examined (Fig. 1C), an increase of 5 times was observed when pH was lowered from 8.5 to 7.0, though values were little affected in the pH range 8.5 to 10. Since the titrated groups for the substrate would be outside the experimental pH range of these studies, this datum suggests that protonation of an enzyme residue with a pK around 7.8 impairs the establishment of the enzyme-substrate complex, probably a histidine imidazolium.

The effects of ionic strength on YFV NS2B/NS3 proteolytic activity against the peptide Abz-AKRRSQ-EDDnp were explored by adding NaCl up to 100 mM (Fig. 2). It is noteworthy that the observed decrease in k_{cat}/K_m values resulted from the systematic increase in the K_m values with the increase in NaCl concentration, particularly in the range 1 to 10 mM. In contrast, the k_{cat} value was only impaired in salt concentrations above 10 mM. This high sensitivity of the protease to salt concentration is also in agreement with data obtained with DV NS2B/NS3 protease [13], and with the previously obtained with YFV NS2B/NS3 using small MCA substrates [7].

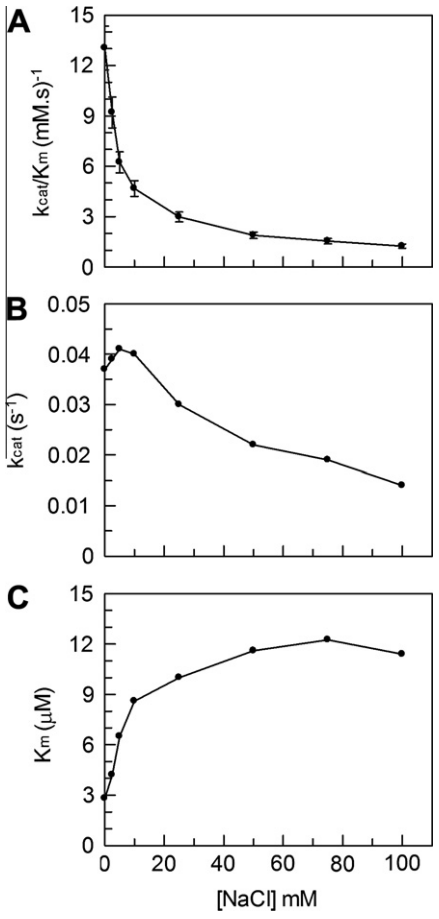

Fig. 2. NaCl effect on k_{cat}/K_m (A), k_{cat} (B) and K_m (C) for YFV NS2B/NS3 hydrolysis of substrate Abz-AKRRSQ-EDDnp. The reactions were carried out under Michaelis–Menten conditions as described in Materials and Methods.

3.2. Hydrolysis of peptide substrates based on polyprotein cleavage sites by YFV NS2B/NS3 protease

The sequence of the FRET peptides based on the polyprotein cleavage sites and their kinetic parameters for hydrolysis by YFV NS2B/NS3 protease are shown in Table 1. All the hydrolyzed peptides were analyzed by LC/MS and the sites of cleavage were found to be the same as those in the polyprotein, indicating that the synthetic peptides containing Abz and EDDnp did not introduce restrictions or different interactions with the viral protease.

Interestingly, though the seven hydrolyzed synthetic peptides had the same Arg-Arg pair interacting at S2-S1 position followed by a small residue (Ala, Gly, Ser – exception is Val in peptide 6), the efficiency of hydrolysis among then varied by more than two orders of magnitude. Peptide 1, corresponding to C_{int} sequence, which contains a sequence of four basic residues in tandem in the non-prime side, showed the highest kinetic parameters values for hydrolysis. By comparing the k_{cat}/K_m value, it was hydrolyzed about 6 times more efficiently than peptides 3 (NS2B/NS3) and 7 (NS4B/NS5) and 20 times or higher more efficiently than peptides 4 ($NS3_{int}$) and 6 (NS4A/NS4B). Peptide 2 (NS2A/NS2B) and 5 (NS3/NS4) were poorly hydrolyzed, showing both higher K_m and low k_{cat} values than the other polyprotein derived peptides.

Since secondary structure studies using peptide substrates have revealed that peptide with more β-sheet like structure tend to react fast with proteolytic enzymes [26], the possible relationship between YFV NS3 activity and the secondary structure of the polyprotein based peptides was investigated by far-UV CD spectra (data not shown). The secondary structure contents were calculated using CDNN software (Applied Photophysics copyright Gerald Böhm 1997) and summarized in table 2. However, while the CD spectra indicate that each peptide forms more or less sheet structures, no correlation between secondary structure and catalytic activity was observed. All together, it is clear that the hydrolytic behavior of YFV NS2B/NS3 protease is deeply affected by the nature of the amino acids others than at P2-P′1, a secondary aspect on DV and WNV processing.

Recently, Condotta and colleagues [27] found discrepancies in the relative catalytic efficiencies of WNV processing sites analyzed *in vitro* and in cell based assay system, suggesting that the membrane microenvironment and/or the other nonstructural protein domains not examined *in vitro* (i.e., NS3 helicase, NS2B N-terminal domain, and NS2B C-terminal domain) do alter the flaviviral NS2B-

Table 1
Kinetic parameters for the hydrolysis by YFV NS2B/NS3 of synthetic FRET peptides substrates based on polyprotein cleavage sites.

Peptide No.	Sequence Abz-peptidyl-EDDnp	Cleavage in polyprotein	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM.s^{-1}$)
1	SSRKRR↓SHDVLTQ	C_{int}	0.069 ± 0.006	2.2 ± 0.2	31.4
2	RIFGRR↓SIPVNEQ	NS2A/NS2B	0.005 ± 0.0005	20 ± 3	0.25
3	VRGARR↓SGDVLWQ	NS2B/NS3	0.041 ± 0.001	8.5 ± 1.8	4.8
4	SAAQRR↓GRIGRNQ	$NS3_{int}$	0.016 ± 0.0001	8.8 ± 0.15	1.8
5	FAEGRR↓GAAEVLQ	NS3/NS4	0.001 ± 0.0001	15 ± 1	0.07
6	KLAQRR↓VFHGVAQ	NS4A/NS4B	0.003 ± 0.0003	2.7 ± 0.7	1.1
7	MKTGRR↓GSANGKQ	NS4B/NS5	0.012 ± 0.0007	2.5 ± 0.3	4.8

Hydrolysis conditions: The proteases were incubated in 50 mM Tris buffer (pH 9.0, 20% glycerol) for 2 min at 37 °C and the reaction started by addition of the substrate.

Table 2
Calculated secondary structure contents for the polyprotein based peptide substrates.

Peptide No.	Sequence Abz-peptidyl-EDDnp	Helix	Sheet	Turn	Other
1	SSRKRRSHDVLTQ	5	14	32	49
2	RIFGRRSIPVNEQ	6	21	29	42
3	VRGARRSGDVLWQ	5	30	23	38
4	SAAQRRGRIGRNQ	6	29	26	37
5	FAEGRRGAAEVLQ	6	25	26	41
6	KLAQRRVFHGVAQ	4	20	26	45
7	MKTGRRGSANGKQ	5	21	27	44

NS3protease enzymatic properties in host cells. However, it is meaningful that the results found here using FRET substrates *in vitro* (Table 1) parallel to those obtained by Chambers et al. (1991) [4] via a vaccinia virus-T7 transient expression system: cleavage at the NS2B-NS3 and NS4B-NS5 sites is relatively efficient when compared with trans cleavage at the NS2A-NS2B and NS3-NS4 sites (the C_{int},$NS3_{int}$ and YF sites were not analyzed).

3.3. Hydrolysis of FRET hexa/heptapeptides based on Arg-ArgSer motif

The exclusive presence of Arg-Arg pair in YFV polyprotein processing sites – a unique feature among flaviviruses – prompt us to analyze the influence of combinations of Arg and Lys basic pairs on YFV NS2B/NS3 catalysis. For that, two series of small FRET peptides containing all possible combinations of three and two basic amino acids in tandem arrangement flanked by small side chain amino acids were assayed for hydrolyses by YFV NS2B/NS3 protease and the results are shown at Tables 3 and 4. In general, peptides containing three natural basic amino acids were hydrolyzed more efficiently than those containing two natural basic amino acids. It should be pointed that all the peptides were susceptible to hydrolysis only at the carboxyl side of the last basic amino acid (lysine or arginine) of the peptide sequence.

3.4. Abz-AKRRSQ-EDDnp analogues

Table 3 shows kinetic parameters for seven peptides derived from the Abz-AKRRSQ-EDDnp peptide. Peptides 16 (Abz-ARKK↓SQ-EDDnp) and 18 (Abz-AKRK↓SQ-EDDnp) were hydrolyzed with higher k_{cat}/K_m values mainly by the lower K_m values. Peptides 12 (Abz-ARRR↓SQ-EDDnp), 13 (Abz-AKRR↓SQ-EDDnp) and 19 (Abz-AKKR↓SQ-EDDnp) were also well hydrolyzed, though with higher K_m values than peptides 16 and 18.

It is clear from Table 3 data that the presence of Arg at P_1 position was related to significative increase in k_{cat} value in relation with those analogues peptides with Lys at P_1. On the other hand, peptides containing Lys possess lower K_m. When comparing analogues peptides with Arg or Lys at P_2, it seems that Arg was related to a two to six times increase in k_{cat} and a small reduction on K_m (exceptions are Abz-AKXR↓SQ-EDDnp and Abz-ARXK↓SQ-EDDnp (x = Arg or Lys) – the first pair showed higher k_{cat} with Lys at P_2 and the second pair an extreme low K_m with Lys). The preference of the S_2 subsite of YFV protease for Arg over Lys is confirmed by the one order of magnitude increase in the efficiency of hydrolysis of peptide Abz-KRR↓SSKQ-EDDnp over Abz- KKR↓SSKQ-EDDnp (k_{cat}/K_m values of 11.9 and 1.0 $(mM.s)^{-1}$, respectively).

Table 3
Kinetic parameters for hydrolysis by YFV NS2B/NS3 of model FRET peptides containing three basic amino acids in tandem.

Peptide No.	Sequence Abz-peptidyl-EDDnp	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m $(mM.s)^{-1}$
8	AKRR↓SQ	0.026 ± 0.004	2.0 ± 0.2	13.00
9	ARRR↓SQ	0.12 ± 0.01	9.6 ± 1.2	12.50
10	ARKR↓SQ	0.060 ± 0.005	14.7 ± 1.8	4.08
11	ARRK↓SQ	0.005 ± 0.0009	1.5 ± 0.2	3.33
12	ARKK↓SQ	0.002 ± 0.0003	0.13 ± 0.05	18.16
13	AKKK↓SQ	0.004 ± 0.0002	6.6 ± 0.6	0.60
14	AKRK↓SQ	0.012 ± 0.004	0.6 ± 0.2	20.00
15	AKKR↓SQ	0.097 ± 0.007	7.6 ± 0.2	12.80
16	KRR↓SSKQ	0.025 ± 0.002	2.1 ± 0.1	11.9
17	KKR↓SSKQ	0.012 ± 0.001	12 ± 1	1.00

The arrows indicate the cleavage sites, and hydrolysis conditions are as described in Table 1.

Table 4
Kinetic parameters for hydrolysis by YFV NS2B/NS3 of model FRET peptides containing two basic amino acids in tandem.

Peptide No.	Sequence Abz-peptidyl-EDDnp	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m $(mM.s)^{-1}$
18	AGRR↓SAQ	0.061 ± 0.008	20 ± 3	3.04
19	AGKK↓SAQ	0.001 ± 0.0001	8.7 ± 1.3	0.11
20	AGRK↓SAQ	0.005 ± 0.0003	5.1 ± 0.8	1.0
21	AGKR↓SAQ	0.010 ± 0.002	7.3 ± 0.005	1.37

Hydrolysis conditions are as described in Table 1.

3.5. Abz-AGRRSAQ-EDDnp analogues

The kinetic parameters for the hydrolysis by YFV NS2B/NS3 of the Abz-AGRRSAQ-EDDnp analogues are shown at Table 4. Similar as observed above, peptides containing Arg at P_1 position showed one order magnitude increase in k_{cat} while a tendency of reduced K_m in peptides containing Lys at P_1 occurred only in the Abz-AGRX-SAQ-EDDnp pair (similar K_m values were obtained within the pair Abz-AGKXSAQ-EDDnp). Higher k_{cat} were also observed in P_2 Arg peptides, though opposite effects were observed in K_m.

In conclusion, we have determined the kinetic parameters of hydrolysis of YFV NS2B-NS3 protease against FRET substrates mimicking the prime and non-prime region of the natural polyprotein cleavage sites, and the large variation on catalytic efficiency supports the notion that substrate interactions others than on P2-P′1 are critical in mediating the selective substrate specificity. The results obtained with peptides containing two and three basic amino acids in tandem indicate that, despite the homogeneity on natural substrates, the YF NS2B-NS3 protease cleaves substrates containing all possible arrangements of basic amino acids at P3-P1, though Arg and Lys residues produced distinct effects on k_{cat}/K_m.

The parallel of our data with those obtained *in vivo* by Chambers et al. (1991) [4] restrain the idea that these sites co-evolved with the NS2B/NS3 protease to promote highly efficient hydrolysis. While further studies are necessary to understand the biological meaning of the unique occurrence of Arg-Arg pair in YFV processing sites, we believe that our data represent a valuable biochemical resource in the characterization of YFV NS2B/NS3 protease.

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