Controlled Peptide Solvation in Portion-Mixing Libraries of FRET Peptides: Improved Specificity Determination for Dengue 2 Virus NS2B-NS3 Protease and Human Cathepsin S

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The solubility of peptides in aqueous buffers used for the enzyme assays is a common limitation for all peptide libraries. In principle, the more water-soluble peptides are, the more susceptible they will be to peptidase hydrolysis. We have demonstrated that this bias can be circumvented in a portion-mixing fluorescence resonance energy transfer (FRET) peptide library by introducing k (lysine in the D-form) in both termini of the peptides. This more solvated library and another one without the k were assayed using trypsin and chymotrypsin as standard peptidases with high selectivity for R and K and for hydrophobic F and Y, respectively. Significantly improved consistency of the information on substrate profiles was obtained from the solvated library. The influence of improved solvation on substrate specificity determination was successfully demonstrated by the difference in specificity observed between the two libraries employing the human cathepsin S (accepts acidic, basic, or neutral amino acids at P_1 position) and Dengue 2 virus NS2B-NS3 protease (high specificity to the pair of basic amino acids K−R, R−R, or Q−R/K at P_2−P_1 positions). In conclusion, hydration of the peptides has a major influence on protease processing, and this bias can be reduced in bound peptide libraries, improving reliability.

Introduction

Combinatorial peptide library approaches have been widely used in systematic assays of individual peptides for determination of protease substrate specificity. Combinatorial peptide library methods involve synthesis of arrays, mixtures, or assemblies of protease substrates in a suitable format for high-throughput screening to identify the optimal substrate sequences. A plethora of methodologies have been described, and the substrate libraries may conveniently be obtained by either biochemical or synthetic procedures. The biochemical methods involve the display of the peptide libraries on filamentous phage and the identification of the best substrates by molecular biology tools including the cleavage site determination in each substrate.[1−4] Synthetic support-bound peptide libraries have been prepared by the process of split-combine synthesis, which results in a single peptide sequence on each of the resin beads. This is a random synthetic library approach that results in one-bead-one structure libraries, and it was extensively reviewed by Lebl et al.,[5] where all the references to the papers of the pioneers in the field can be found. Synthetic peptide libraries can contain thousands of compounds, and the identification of the substrates and the cleavage sites require analytical methods such as Edman degradation,[6,7] mass spectrometry,[8,9] or chromatography.[10] Positional-scanning synthetic combinatorial libraries (PS-SCLs) of fluorogenic peptide substrates is a strategy in which each position in the peptide sequence is occupied in turn by a single amino acid residue. The other positions are randomly occupied by one of 20 natural amino acids, and [7-amino-4-methyl]-coumarin (AMC) or [7-amino-4-carbamoyl-methyl]-coumarin (ACC) are positioned at the C-terminal carboxyl group of the peptides.[11,12] This concept was also employed for the study of carboxydipeptidase specificity of cathepsin B[13] and angiotensin converting enzyme,[14] using fluorescence resonance energy transfer (FRET) peptides.

Support-bound FRET peptide libraries[15] have been prepared by the process of split-combine synthesis, which results in a single peptide sequence on each of the resin beads.[16] Partial proteolysis of the substrates bound to the solid support and sequence determination by Edman degradation of the substrates on the most fluorescent beads provides the sequences and the cleavage sites of the optimal substrates.[17−19] Support-bound peptide mimetic inhibitor libraries for proteases have also been developed and were called "combinatorial one-bead two-compound libraries".[20,21]

The solubility of the generated peptides in aqueous buffer for the enzyme assays is a limitation common to all the peptide libraries. In principle, the more water-soluble peptides will be more susceptible to peptidase hydrolysis because of

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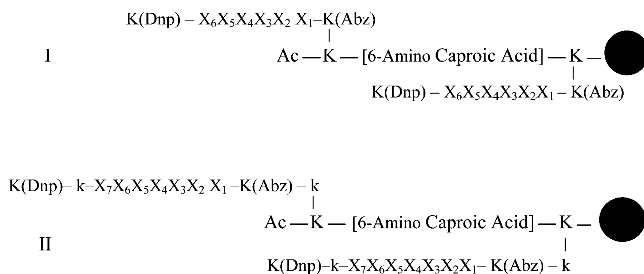

Figure 1. General structures of the libraries I and II.

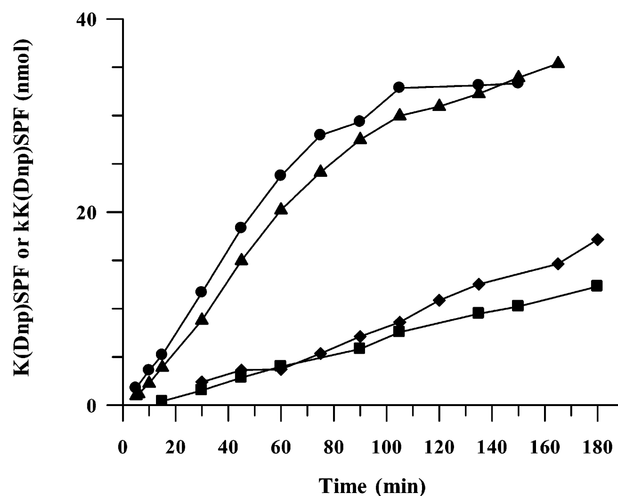

Figure 2. Hydrolysis by chymotrypsin of the peptides K(Dnp)-SPFSASK(Abz) (■) and kK(Dnp)SPFSASkK(Abz) (▲) attached to resin. Results of the same experiment in the presence of 0.01% Tween, for K(Dnp)SPFSASK(Abz) (◆) and for kK(Dnp)SPFSASkK-(Abz) (●). Conditions of hydrolysis: 100 mM Tris HCl, 10 mM $CaCl_2$, pH 8.0, 5 μM TLCK, 1.5 μM chymotrypsin, at 25 °C. K(Dnp)SPF and kK(Dnp)SPF were quantified by HPLC.

their increased exposure to the enzyme in the solvent. It is also reasonable to anticipate that differences in the peptide solubility introduces a bias in the screening of substrates by peptidases that have preferences for bulky hydrophobic amino acids or for those that can accept both hydrophilic and hydrophobic amino acids. To further improve the applications of portion-mixing FRET peptide libraries as previously described by Meldal[15] for endopeptidases and to evaluate the effects of water solvation on the screening of substrates for proteases we synthesized two libraries containing k (lysine in D-form) in both termini of the peptides (hydrophilic library) and compared them with a library without these k residues (hydrophobic library). Figure 1 shows the general structures of the FRET peptide libraries synthesized for this investigation. The amount of peptide in each bead was doubled by the introduction of two K units separated by 6-amino caproic acid. 2-Aminobenzoic acid (Abz) and *N*-[2,4-dinitrophenyl] (Dnp) were used as FRET donor and acceptor groups, respectively. These libraries were initially assayed with trypsin and chymotrypsin as standard peptidases that have high selectivity for positively charged (R and K) and for hydrophobic (F and Y) amino acids, respectively. To determine the consistency of the information obtained about substrate specificity by protease incubation of the hydrophobic and hydrophilic libraries, respectively, these were assayed with human cathepsin S and Dengue 2 virus NS2B-NS3 protease, which both have particular specificities. Dengue 2 virus NS2B-NS3 protease has high specificity to a pair of basic amino acids (K−R or R−R)[22−24] and human cathepsin S accepts acidic, basic, or neutral amino acids at the P_1 position[25−28] (nomenclature of Schechter and Berger).[29]

Results and Discussion

Resins and Linkers. Solid-phase chemistry and biochemistry are both very dependent on the composition and physical properties of the polymer matrix. The polystyrene-based solid support is a classic hydrophobic resin that is inappropriate for assays that require interactions of proteins with peptides linked to the resins. Thus highly solvated hydrophilic polyethylene glycol (PEG)-based resins have been developed, including the amino-functionalized polyethyleneglycol-polyamide copolymer, PEGA, resin.[30−32] The amphiphilic nature of PEG provides this kind of resin with the property of superior solvation and permeability in both polar and nonpolar solvents that permits the synthesis of peptides and their exposure in aqueous buffers as substrates for peptidases in protease assays.[17−19] The fluorescence-quenched libraries, the structures of which are shown in Figure 1, were synthesized on $PEGA_{1900}$ (1 g, 0.11 mmol/g). Six positions (X_1−X_6) in library I and seven positions (X_1−X_7) in library II were randomized using all the natural L amino acids. The peptides were flanked by Abz attached to K side chain [K(Abz)], which is the fluorescence donor, and by Dnp also attached to a K side chain [K(Dnp)] that is the fluorescence acceptor. The sequence acetyl-K-[6-amino caproyl]-K was incorporated as linker of the peptides to double the resin loading (0.22 mmol/g). FRET peptides were assembled on the K side chain, and the 6-amino caproyl residue served to increase the distance between the peptides in each bead, which improved the brightness of the beads upon enzymatic cleavage of the peptides. In preliminary experiments, where only Acetyl-K−K was used as linker of the peptides to the resin, it was observed that the beads were dark and difficult to manipulate. The k residue was introduced at the N- and C-termini in the peptides of library II as shown in the Figure 1. The position of k after K(Dnp) was chosen after preliminary experiments had demonstrated a preference by chymotrypsin and papain for K(Dnp) at the P_2 position in all L-amino acid substrates. The second k residue was positioned after K(Abz) to avoid resistance of cleavage at carboxyl group of X_1 residues because most of the proteases we have assayed do not cleave at the amino end of amino acids in the D-form.

Activity of Chymotrypsin on Peptide Attached in Beads and in Solution. The peptides K(Dnp)SPFSASK(Abz)-NH_2 and kK(Dnp)SPFSASkK(Abz)-NH_2 were synthesized as free peptides and also synthesized permanently attached to $PEGA_{1900}$ resin. Portions (5 mg) of the resins containing each one of these peptides were incubated with 70 nmol of chymotrypsin in 3 mL of a solution containing 100 mM Tris-HCl and 10 mM $CaCl_2$, pH 8.0. Aliquots of the supernatant buffer without resin were analyzed by HPLC, and the amount of K(Dnp)SPF-OH or kK(Dnp)SPF-OH was quantified. Figure 2 shows the progress of hydrolysis during 180 min, and the peptide containing the two k residues is hydrolyzed

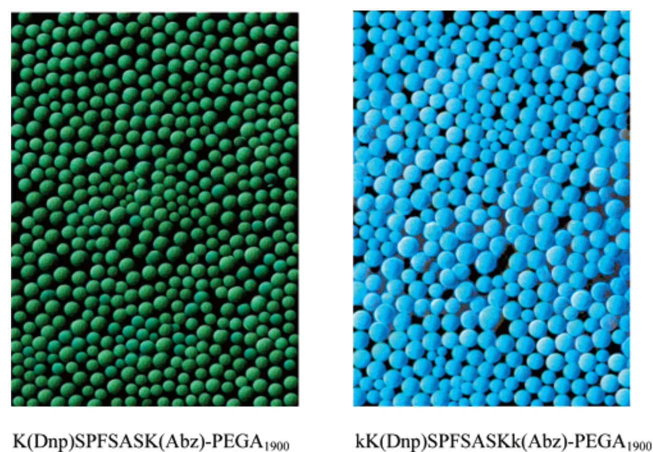

Figure 3. Beads of $PEGA_{1900}$ resin containing the peptides K(Dnp)-SPFSASK(Abz) and kK(Dnp)SPFSASkK(Abz) after 2 h of incubation with chymotrypsin observed under a fluorescence microscope.

Table 1. Kinetic Parameters for Chymotrypsin Hydrolysis in Solution after Cleavage of the Resin of the Resin-Bound Peptides Used in the Solid-Phase Experiments Shown in Figure 2[a]

sequences	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m $(s\ mM)^{-1}$
K(Dnp)-S-P-F-S-A-S-K(Abz)NH_2	1.8 ± 0.1	1.20 ± 0.05	1400 ± 140
k-K(Dnp)-S-P-F-S-A-S-k-K(Abz)NH_2	2.8 ± 0.2	3.3 ± 0.1	800 ± 80

[a] Conditions of hydrolysis: 100 mM Tris HCl, 10 mM $CaCl_2$, pH 8.0, 5 μM TLCK, 4.4 nM chymotrypsin, 37 °C.

faster. This difference in the velocity of hydrolysis can be also clearly observed in Figure 3 that shows the beads after 120 min of incubation with 70 nM of chymotrypsin and that the beads containing K(Dnp)SPFSASK(Abz) presented almost no fluorescence compared to the beads with kK(Dnp)-SPFSASkK(Abz). A slight but significant improvement in the hydrolysis of both peptides attached to beads was observed in the presence of 0.01% Tween as detergent (Figure 2).

The same peptides were also assayed in solution with chymotrypsin at lower peptide concentration but otherwise under identical conditions, and the kinetic parameters for their hydrolysis are shown in Table 1. The difference for the hydrolysis rates of the two peptides was small, but significant, because the k_{cat}/K_m value for the hydrolysis of K(Dnp)-SPFSASK(Abz)-NH_2 was twice that of kK(Dnp)SPFSASkK-(Abz)-NH_2. This hydrophilic peptide was approximately 10 times more soluble than K(Dnp)SPFSASK(Abz)-NH_2 as determined under the conditions used for measurement of the kinetics of hydrolysis. Therefore, the faster hydrolysis by chymotrypsin of kK(Dnp)SPFSASkK(Abz) compared to that of K(Dnp)SPFSASK(Abz) when both peptides were bound to resin at 0.22 mmol/g resin could be related to the higher solvation and thus better exposure of kK(Dnp)-SPFSASkK(Abz)-NH_2 to the protease in water.

Screening of Peptide Libraries with Chymotrypsin and Trypsin. Figure 4 shows samples of libraries I and II after treatment with chymotrypsin and trypsin. Very few fluorescent beads were detected in library I compared to library II after treatment with chymotrypsin. In contrast, apparently

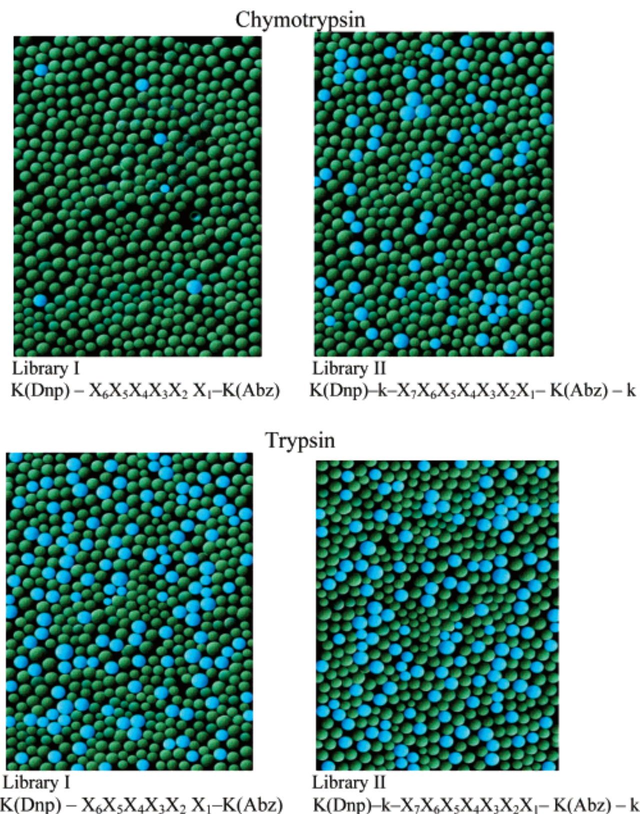

Figure 4. Beads of $PEGA_{1900}$ resin from the libraries I and II after the incubation with trypsin (15 h) and chymotrypsin (20 h) observed under the fluorescence microscope.

Table 2. Substrates for Chymotrypsin Obtained from Library I (K(Dnp)$X_6X_5X_4X_3X_2X_1$K(Abz)) after Incubation with the Peptidase[a]

K(Dnp)-R-M-Y↓K-I-R-K(Abz)
K(Dnp)-P-L-R-F↓K-R-K(Abz)
K(Dnp)-R-R-W↓T-V-R-K(Abz)
K(Dnp)-K-S-Q-V-Y↓R-K(Abz)

[a] Conditions of hydrolysis: 50 mg resin, 100 mM Tris HCl, 10 mM $CaCl_2$, pH 8.0, 5 μM TLCK, 4.4 nM chymotrypsin, 25 °C. The fluorescent beads were collected after 20 h of reaction.

no differences could be observed between the two libraries after treatment with trypsin. Table 2 shows the peptides present in four selected fluorescent beads after treatment of library I with chymotrypsin. These peptides contain two or three basic amino acids and only one aromatic residue, which is the specificity at the cleavage site of chymotrypsin. Table 3 shows the sequences identified from library I in presence of 0.01% Tween, and approximately three times more fluorescent beads were identified, isolated, and sequenced. Most of the identified peptides in this second screening in the presence of detergent also contain one aromatic residue and two or more basic or acidic residues. This moderated improvement on the detection of more peptides as substrates of chymotrypsin resulting from the detergent effect of Tween is in accordance with the slight increase of hydrolysis rate observed for the peptides K(Dnp)SPFSASK(Abz) and kK-(Dnp)SPFSASkK(Abz) bound to the beads (Figure 2).

The peptides susceptible to chymotrypsin identified from library II are shown in Table 4. The substrates contain two or more aromatic or hydrophobic amino acids, and some of

Table 3. Substrates Obtained for Chymotrypsin from Library I (K(Dnp)$X_6X_5X_4X_3X_2X_1$K(Abz)) after Incubation with the Peptidase in the Presence of 0.01% Tween[a]

K(Dnp)-R-W↓T-A-F↓K-K(Abz)
K(Dnp)-T-Y↓K-P-F↓M-K(Abz)
K(Dnp)-F↓E-H-W↓L-V-K(Abz)
K(Dnp)-I-L-F↓Y↓I-T-K(Abz)
K(Dnp)-K-T-I-R-Y↓G-K(Abz)
K(Dnp)-L-N-T-I-F↓R-K(Abz)
K(Dnp)-S-K-G-N-Y↓K-K(Abz)
K(Dnp)-W↓A-D-K-R-L-K(Abz)
K(Dnp)-E-F↓D-K-Q-E-K(Abz)
K(Dnp)-K-F↓H-I-Q-K-K(Abz)
K(Dnp)-R-K-Q-K-I-W↓K(Abz)
K(Dnp)-P-L-F↓P-E-D-K(Abz)

[a] Conditions of hydrolysis: resin (50 mg), 100 mM Tris HCl, 10 mM $CaCl_2$ 0,01% Tween, pH 8.0, 5 μM TLCK, 4.4 nM chymotrypsin, 25 °C. The fluorescent beads were collected after 20 h of reaction.

Table 4. Substrates Obtained for Chymotrypsin from Library II (K(Dnp)k$X_7X_6X_5X_4X_3X_2X_1$K(Abz)k) after Incubation with the Peptidase[a]

cleavage at 2 or 3 peptide bonds
K(Dnp)-k-K-F↓F↓K-W↓T-A-K(Abz)-k
K(Dnp)-k-S-V-K-Y↓Y↓G-W↓K(Abz)-k
K(Dnp)-k-Q-A-Y↓Y↓M-Y↓K-K(Abz)-k
K(Dnp)-k-Q-K-F↓L-Y↓Y↓R-K(Abz)-k
K(Dnp)-k-T-P-R-F↓Y↓K-Y↓K(Abz)-k
K(Dnp)-k-S-A-M-T-T-Y↓Y↓K(Abz)-k
K(Dnp)-k-Q-K-R-Y↓F↓R-V-K(Abz)-k
K(Dnp)-k-A-R-R-M-L-F↓F↓K(Abz)-k
K(Dnp)-k-G-M-K-Y↓F↓R-G-K(Abz)-k
K(Dnp)-k-I-V-T-F↓V-G-F↓K(Abz)-k
K(Dnp)-k-W↓F↓K-E-R-Q-K-K(Abz)-k
cleavage at 1 peptide bond
K(Dnp)-k-R-G-Q-R-A-V-Y↓K(Abz)-k
K(Dnp)-k-Q-R-F↓G-G-M-A-K(Abz)-k

[a] Conditions of hydrolysis: resin (50 mg), 100 mM Tris HCl, 10 mM $CaCl_2$, pH 8.0, 5 μM TLCK, 4.4 nM chymotrypsin, 25 °C. The fluorescent beads were collected after 20 h of reaction.

them do not present any charged side chain residues. Thus, chymotrypsin selected the most hydrophilic sequences in hydrophobic library I, and this bias was reduced in library II. In this library, the peptides with more hydrophobic amino acids were selected as substrate by chymotrypsin and presumably more accurately reflect the genuine specificity of the enzyme.

More than forty fluorescent beads were isolated from library I after treatment with trypsin, and then the peptides were sequenced (Table 5). Some of the identified sequences were synthesized but using Abz attached to the amino group of the first amino acid and Q-EDDP (glutamyl-*N*-[ethylenediamine] 2,4-dinitrophenyl) attached to C-terminal carboxyl group, that is, the peptide K(Dnp)KTRRSTK(Abz) was synthesized as Abz-KTRRST-Q-EDDnp and assayed as such in solution. The k_{cat}/K_m values for the hydrolysis of these selected peptides are shown in Table 5. Substrates were identified very efficiently, although the most-efficient hydrolyzed peptides were often cleaved at more than one site. The worst substrates were those hydrolyzed at R when this amino acid was located at N-terminal end as in Abz-RHGTHF-Q-EDDnp ($k_{cat}/K_m = 200$ s^{-1} mM^{-1}) and Abz-RGTANA-Q-EDDnp ($k_{cat}/K_m = 70$ s^{-1} mM^{-1}) (Table 6). In these cases, the lack of amino acids at non-prime sites of the substrates reduces the substrate–enzyme interaction.

Table 6 shows the sequences of a sample of the fluorescent beads (approximately 3% of all fluorescent beads) of library II after treatment with trypsin. It is worth mentioning that these sequences have a high frequency of the hydrophobic amino acids I, V, and Y, even though trypsin has primary selectivity for the hydrophilic basic amino acids R and K in P_1. Thus, in library II, even trypsin was able to select substrates with more hydrophobic amino acids in the secondary binding sites.

Screening of Peptide Libraries with Human Cathepsin S. The peptides identified as substrates for human cathepsin S in the screening of libraries I and II are shown in Table 7. It is noteworthy that the peptides from library I were hydrolyzed only at basic amino acids or at the acidic E residue. In contrast, the peptides from library II were hydrolyzed at hydrophobic amino acids, V, I, F, Y, and A, or at the E residue. Similar to the activities of chymotrypsin and trypsin on libraries I and II, the substrates for cathepsin S identified in library I contain more hydrophilic sequences than those identified from library II. Some of the identified sequences were synthesized using Abz/Q-EDDP as the donor–acceptor pair. The three best substrates for cathepsin S were Abz-KRWRLE-Q-EDDnp, Abz-NGVRLN-Q-EDDnp, and Abz-NLRTIN-Q-EDDnp, for which k_{cat}/K_m values are presented in Table 7. All these peptides were hydrolyzed at R residue in the P_1 position; however, the peptide Abz-SMVRKA-Q-EDDnp was hydrolyzed at the R–K bond with a significantly lower k_{cat}/K_m value. Most of the peptides identified from library II were hydrolyzed at more than one peptide bond. The preference of cathepsin S for basic residues K and R was observed in prevoiusly reported screening of PS-SCLs of fluorogenic peptide substrates.[27] However, in the cleavage of the oxidized insulin β-chain by cathepsin S, the preferentially hydrolyzed bonds were E13–A14, L17–V18, and F23–Y26,[25] and in peptides derived from MHC class II-associated invariant chain, the preferred amino acids at P_1 position were A, E, G, K, L, Q, and S.[27] The specificity of subsite S_2 of cathepsin S is restricted to aliphatic branched side chain amino acids, such as L, V, and I, and for M, the most frequent amino acids present in the P_2 position of all substrates identified in libraries I and II. This restricted preference of the S_2 subsite of cathepsin S was previously observed using different approaches.[26–28] The high frequency of hydrophobic (L, I) and small side chain amino acids (A and S) at position P_1' of the substrates in Table 7 is in agreement with previously reported S_1' specificity in the series of substrates Dns-F-R-X-W-A, where X represents different natural amino acids.[33] These results with cathepsin S demonstrated that the assay of both libraries I and II gave better and more reliable analysis of its known substrate preferences than the alternative methods.[27] The obtained results, particularly that of library II, were in accordance with the previously reported biological preferences of cathepsin S with only one type of S_1 subsite specificity.[28]

Screening of Peptide Libraries with Dengue 2 Virus NS2B-NS3 Protease. The peptides identified as substrates

Table 5. Substrates Obtained for Trypsin from Library I (K(Dnp)$X_6X_5X_4X_3X_2X_1$K(Abz)) after Incubation with the Peptidase[a]

cleavage at 2 or 3 peptide bonds	k_{cat}/K_m (s mM)$^{-1}$	cleavage at 1 peptide bond	k_{cat}/K_m (s mM)$^{-1}$
K(Dnp)-R↓S-K↓G-K↓H-K(Abz)		K(Dnp)-L-P-R↓H-L-P-K(Abz)	13 000
K(Dnp)-K↓T-R↓R↓S-T-K(Abz)	59 000	K(Dnp)-I-T-H-G-R↓Q-K(Abz)	4000
K(Dnp)-R↓R↓G-R↓F-D-K(Abz)		K(Dnp)-M-Y-K↓Q-M-Y-K(Abz)	3000
K(Dnp)-R↓I-R↓K↓G-I-K(Abz)		K(Dnp)-R↓G-L-G-Q-I-K(Abz)	
K(Dnp)-R↓K↓Q-K↓V-W-K(Abz)		K(Dnp)-L-S-K↓G-L-S-K(Abz)	2000
K(Dnp)-G-R↓R↓A-G-L-K(Abz)	42 000	K(Dnp)-R↓A-F-N-F-N-K(Abz)	
K(Dnp)-N-Y-F-R↓K↓G-K(Abz)	35 000	K(Dnp)-P-H-H-A-G-R↓K(Abz)	
K(Dnp)-H-K↓Y-R↓A-H-K(Abz)	22 000	K(Dnp)-Y-L-G-R↓G-N-K(Abz)	2000
K(Dnp)-R↓K↓I-M-P-P-K(Abz)		K(Dnp)-D-G-D-N-A-R↓K(Abz)	
K(Dnp)-R↓K↓G-T-H-F-K(Abz)		K(Dnp)-I-I-N-I-F-K↓K(Abz)	
(Dnp)-Y--K↓R↓A-N-T-K(Abz)	19 000	K(Dnp)-R↓H-G-T-H-F-K(Abz)	200
K(Dnp)-G-R↓L-K↓E-L-K(Abz)		K(Dnp)-R↓G-T-A-N-A-K(Abz)	70
K(Dnp)-P-R↓L-K↓H-N-K(Abz)			
K(Dnp)-V-R↓R↓V-N-T-K(Abz)	19 000		
K(Dnp)-R↓H-F-K↓G-F-K(Abz)			
K(Dnp)-R↓Q-Q-K↓G-F-K(Abz)			
K(Dnp)-M-P-K↓A-R↓N-K(Abz)			
K(Dnp)-K↓G-F-T-A-R↓K(Abz)			
K(Dnp)-N-K↓L-H-G-R↓K(Abz)			
K(Dnp)-S-R↓M-F-H-R↓K(Abz)			
K(Dnp)-P-R↓N-P-R↓G-K(Abz)			
K(Dnp)-M-R↓G-H-R↓H-K(Abz)			
K(Dnp)-F-N-R↓A-R↓N-K(Abz)			
K(Dnp)-V-R↓T-P-L-R↓K(Abz)			
K(Dnp)-R↓L-E-R↓E-M-K(Abz)			
K(Dnp)-G-H-R↓S-G-R↓K(Abz)			

[a] Conditions of hydrolysis: (a) for solid-phase library screening, resin (50 mg), 100 mM Tris HCl, 10 mM Ca^{2+}, pH 8.0, 5 μM TPCK, 4.2 nM trypsin, 25 °C. The fluorescent beads were collected after 15 h of reaction. For k_{cat}/K_m determination in solution: 100 mM Tris HCl, 10 mM $CaCl_2$, pH 8.0, 5 μM TPCK, 27 nM trypsin at 37 °C. (b) For k_{cat}/K_m values, they were obtained in solution with peptides synthesized using the same sequence, but Abz was attached to the amino group of the first amino acid and Q-EDDP (glutamyl-*N*-[ethylenediamine] 2,4-dinitrophenyl) was attached to C-terminal carboxyl group, for example, the peptide K(Dnp)-K-T-R-R-S-T-K(Abz) was synthesized and assayed in solution as Abz-K-T-R-R-S-T-Q-EDDnp.

Table 6. Substrates Obtained for Trypsin from Library II (K(Dnp)k$X_7X_6X_5X_4X_3X_2X_1$K(Abz)k) after Incubation with the Peptidase[a]

K(Dnp)-k-N-R↓H-R↓S-K↓S-K(Abz)-k
K(Dnp)-k-G-Y-K↓R↓I-R↓I-K(Abz)-k
K(Dnp)-k-K-G-R↓R↓I-A-R↓K(Abz)-k
K(Dnp)-k-S-R↓G-G-R↓G-K↓K(Abz)-k
K(Dnp)-k-R↓H-P-H-K↓A-R↓K(Abz)-k
K(Dnp)-k-Y-Q-R↓Y-R↓Q-V-K(Abz)-k
K(Dnp)-k-K↓V-Q-V-K↓I-A-K(Abz)-k
K(Dnp)-k-K↓H-K↓A-T-A-V-K(Abz)-k
K(Dnp)-k-K↓V-V-T-V-T-K↓K(Abz)-k
K(Dnp)-k-R↓I-A-I-I-A-A-K(Abz)-k

[a] Conditions of hydrolysis: 50 mg resin, 100 mM Tris HCl, 10 mM $CaCl_2$, pH 8.0, 5 μM TPCK, 4.2 nM trypsin, 25 °C. The fluorescent beads were collected after 15 h of reaction.

for Dengue 2 virus NS2B-NS3 protease in the screening of libraries I and II are shown in Table 8. Most of the selected beads contain peptides hydrolyzed after a pair of basic amino acids, which is in accordance with the recently reported specificity of Dengue 2 virus NS2B-NS3 protease.[22−24] We could not detect any significant difference on the solubility characteristics of the amino acids that compose the peptides identified as substrates of NS2B-NS3 protease in the two libraries. This result can be related to the natural selectivity of NS2B-NS3 protease for hydrophilic basic sequences in the dengue polyprotein.[24] Then, this protease only selected in library I the peptides with basic amino acid sequences that are already more soluble and exposed to solvent. The reliability of these libraries is noteworthy because, taken together, the most frequent sequences of amino acids in the positions P_3 to P_3' corresponds to the sequence T-R/K-R-G-K-R/G that has a reasonable similarity to the sequence T-R-R-G-T-G that span the cleavage site of dengue virus polyprotein at the junction of NS4B−NS5 of the virus polyprotein. Most of the peptides identified in the libraries that were hydrolyzed after just only one basic amino acid revealed Q at P_2 position. This amino acid is also present in the sequence around the cleavage sites NS2B-NS3 (...KRQR↓AGVL...) and internal to NS4A (...EKQR↓TPQD...) of the polyprotein. In addition, the identified substrate, K-R-Q-K↓G-S (Table 8), has a significant similarity to the polyprotein cleavage site NS2B-NS3 (...KRQR↓AGVL...).

Conclusions

Hydration on solid support of the peptides that constitute the substrate libraries has a major influence on protease processing and introduces bias in the selectivity of the proteases toward substrates in resin-bound peptide libraries. We demonstrated that this problem can be at least partially circumvented in portion-mixing FRET peptide libraries by introducing k at the N,C termini of the peptides to provide even hydration and, therefore, a more genuine profile of the protease specificity. This was first investigated for the archetypal proteases trypsin and chymotrypsin. In addition, as demonstrated for cathepsin S and for Dengue 2 virus NS2B-NS3 protease, more detailed information and reliability of peptidase substrate specificity can be obtained by assaying the enzyme with both the hydrophobic library without lys and the hydrophilic with this amino acid. The

Table 7. Substrates Obtained for Human Cathepsin S from Libraries I (K(Dnp)$X_6X_5X_4X_3X_2X_1$K(Abz) and II (K(Dnp)k$X_7X_6X_5X_4X_3X_2X_1$K(Abz)k) after Incubation with the Peptidase[a]

	k_{cat}/K_m (s mM)$^{-1}$
library I K(Dnp)$X_6X_5X_4X_3X_2X_1$K(Abz)	
K(Dnp)-E-L-K↓V-E↓L-K(Abz)	17
K(Dnp)-T-P-E-M-R↓L-K(Abz)	
K(Dnp)-M-Q-E↓M-Q-E-K(Abz)	
K(Dnp)-T-L-E↓M-P-H-K(Abz)	
K(Dnp)-L-E↓S-K-E-S-K(Abz)	
K(Dnp)-K-R-W-R↓L-E-K(Abz)	4350
K(Dnp)-V-G-L-K↓L-N-K(Abz)	
K(Dnp)-A-L-K↓G-V-F-K(Abz)	
K(Dnp)-T-N-P-L-R↓S-K(Abz)	
K(Dnp)-N-L-R↓T-I-N-K(Abz)	536
K(Dnp)-N-G-V-R↓L-N-K(Abz)	1720
K(Dnp)-M-G-L-R↓G-Q-K(Abz)	
K(Dnp)-K-D-T-R↓A-H-K(Abz)	
K(Dnp)-S-M-V-R↓K-A-K(Abz)	60
library II K(Dnp)-k$X_7X_6X_5X_4X_3X_2X_1$K(Abz)-k	
K(Dnp)-k-K-N-I-V↓V-L↓S-K(Abz)-k	
K(Dnp)-k-K-R-V-I↓I↓V↓V-K(Abz)-k	
K(Dnp)-k-P-A-I-A↓A-I-A-K(Abz)-k	
K(Dnp)-k-I-M↓M↓Y-Y↓I-V-K(Abz)-k	
K(Dnp)-k-Y-A-I-Y↓Y↓I-V-K(Abz)-k	
K(Dnp)-k-Y-W-E↓Y-T↓A-T-K(Abz)-k	52
K(Dnp)-k-K-D-M-V↓Y↓V↓T-K(Abz)-k	
K(Dnp)-k-Q-L-F↓E↓A-A-V-K(Abz)-k	21
K(Dnp)-k-I-V↓Y-M-S↓L-D- K(Abz)-k	
K(Dnp)-k-G-A-S-I-I-E↓A-K(Abz)-k	
K(Dnp)-k-T-A-A-L-E↓E-R-K(Abz)-k	79
K(Dnp)-k-R-K-I-F↓F-I-F-K(Abz)-k	
K(Dnp)-k-V-F↓F-A-A-D-V-K(Abz)-k	
K(Dnp)-k-I-V↓V-S-M-H-Q-K(Abz)-k	
K(Dnp)-k-V-V-E↓Q-T-M-T-K(Abz)-k	

[a] Conditions of hydrolysis: (a) for solid-phase library screening, 50 mg resin in 50 mM sodium phosphate, 2.5 mM EDTA, pH 6.5 and 25 nM of cathepsin S that was preactivated with 10 mM dithoerytritol at 25 °C (the fluorescent beads were collected after 7 h of reaction); (b) for k_{cat}/K_m values, they were obtained in solution of 50 mM sodium phosphate, 2.5 mM EDTA, pH 6.5, 12 nM of cathepsin S that was preactivated with 10 mM dithioerytritol, 37 °C. The k_{cat}/K_m values were obtained with the peptides synthesized as described in Table 5.

Table 8. Substrates Obtained for Human Dengue 2 Virus NS2B-NS3 Protease from Libraries I (K(Dnp)$X_6X_5X_4X_3X_2X_1$K(Abz) and II (K(Dnp)k$X_7X_6X_5X_4X_3X_2X_1$K(Abz)k) after Incubation with the Peptidase[a]

library I K(Dnp)$X_6X_5X_4X_3X_2X_1$K(Abz)	library II K(Dnp)-k-$X_7X_6X_5X_4X_3X_2X_1$K(Abz)-k
K(Dnp)-M-K-G-K-R↓Q-K(Abz)	K(Dnp)-k-M-K-F-R-K↓T-T-K(Abz)-k
K(Dnp)-G-K-K↓T-L-R-K(Abz)	K(Dnp)-k-K-K-L-R-R↓T-R-K(Abz)-k
K(Dnp)-E-R-K↓S-A-I-K(Abz)	K(Dnp)-k-K-K↓F-G-W-S-A-K(Abz)-k
K(Dnp)-L-K-R↓G-T-R-K(Abz)	K(Dnp)-k-K-F-R-K↓G-V-R-K(Abz)-k
K(Dnp)-P-K-K↓V-K-R-K(Abz)	K(Dnp)-k-K-K-T-R-R↓S-Q-K(Abz)-k
K(Dnp)-K-Y-H-R-K↓G-K(Abz)	K(Dnp)-k-A-T-R-R↓Q-Y-R-K(Abz)-k
K(Dnp)-I-N-R-R↓H-T-K(Abz)	K(Dnp)-k-Q-K-R-K↓G-V-G-K(Abz)-k
K(Dnp)-K-R-Q-K↓G-S-K(Abz)	K(Dnp)-k-N-T-L-N-Y-R↓S-K(Abz)-k
K(Dnp)-K-A-Q-R↓A-K-K(Abz)	K(Dnp)-k-F-S-N-Y-Q-R↓S-K(Abz)-k

[a] Conditions of hydrolysis: 50 mg resin, in 50 mM Tris, 10 mM NaCl, 20% glycerol, pH 9.0, and 30 nM of Dengue 2 virus NS2B-NS3, 37 °C. The fluorescent beads were collected after 7 h of reaction.

effects of hydrophobic packing in the library I may well represent the situation in the densely packed cellular environment of, for example, cytosol, endo-, or lyzosomes and is not without interest; in fact, the conventional assay of using fluorogenic substrates at high dilution to determine kinetic parameters is quite artificial and could often be misleading with respect to the actual work performed by the protease in its natural habitat.

Experimental Section

All solvents were appropriately distilled before use and DMF-assayed for free amine residues.[34] All Fmoc amino acids were purchased from Calbiochem−Nova Biochem. Trifluoroacetic acid (TFA), anisol, and 1,2-ethanedithiol were from Fluka. Benzotriazole-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) was from Watanabe Chemical Co. PEGA resin (0.1 mmol/g) was from Carlsberg Laboratory, synthesized as previously reported[30−32] The UIPAC nomenclature of 1- and 3-letter codes for the L- and D-amino acids are used, that is, D-amino acids are indicated with a lower-case letter, for example, k for D-lysine.

Peptide Synthesis. The FRET peptides contained *N*-(2,4-dinitrophenyl)-ethylenediamine (EDDnp) attached to Q, a necessary prerequisite for the solid-phase peptide synthesis strategy employed as detailed elsewhere.[35] An automated benchtop simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system from Shimadzu) was used for the solid-phase synthesis of all the peptides by the Fmoc-procedure. Prior to coupling, the Fmoc amino acids (1 equiv) were preactivated with HOBt (1 equiv), TBTU (1 equiv), and NMM (2 equiv) for 5 min. The final, deprotected peptides were purified by semipreparative HPLC using an Econosil C-18 column (10 μm, 22.5 mm × 250 mm) and a two-solvent system: (A) trifluoroacetic acid (TFA)/H_2O (1:1000) and (B) TFA/acetonitrile (ACN)/H_2O (1:900:100). The column was eluted at a flow rate of 5 mL/min with a 10 (or 30) to 50 (or 60)% gradient of solvent B over 30 or 45 min. Analytical HPLC was performed using a binary HPLC system from Shimadzu with a SPD-10AV Shimadzu UV−vis detector and a Shimadzu RF-535 fluorescence detector, coupled to an Ultrasphere C-18 column (5 μm, 4.6 mm × 150 mm) which was eluted with solvent systems A1 (H_3PO_4/H_2O, 1:1000) and B1 (ACN/ H_2O/H_3PO_4, 900:100:1) at a flow rate of 1.7 mL/min and a 10 to 80% gradient of B1 over 15 min. The HPLC column eluates were monitored by their absorbance at 220 nm and by fluorescence emission at 420 nm, upon excitation at 320 nm. The molecular weights and purities of all peptides synthesized were checked by MALDI-TOF mass spectrometry (TofSpec-E, Micromass). The concentrations of the solutions of the substrates were determined by measurement of the absorption of the 2,4-dinitrophenyl group (extinction coefficient at 365 nm being 17.300 M^{-1} cm^{-1}).

Synthesis of Substrate Libraries. The syntheses of libraries were carried out manually as previously described.[21] The libraries were synthesized on 1 g of $PEGA_{1900}$[30,31] resins in a 20-column Teflon synthesis block, using the following orthogonal protected Fmoc amino acids: Fmoc-A-OH, Fmoc-R(PMC)-OH, Fmoc-N(Trt)-OH, Fmoc-G-OH, Fmoc-Q(Trt)-OH, Fmoc-H(Trt)-OH, Fmoc-F-OH, Fmoc-P-OH, Fmoc-S(*t*Bu)-OH, Fmoc-T(*t*Bu)-OH, Fmoc-W(Boc)-OH,

Fmoc-Y(*t*Bu)-OH, Fmoc-V-OH, Fmoc-C(Trt)-OH, Fmoc-D(*t*Bu)-OH, Fmoc-I-OH, Fmoc-E(*t*Bu)-OH, Fmoc-L-OH, Fmoc-K(Boc)-OH, and Fmoc-M-OH. The resin was evenly distributed in the 20 wells of the Teflon synthesis block, and the Fmoc groups were removed. Prior to coupling, the Fmoc amino acids (1 equiv) were preactivated with HOBt (1 equiv), TBTU (1 equiv), and NMM (2 equiv) in DMF (1 mL) for 6 min, and the activated amino acids were added, one to each of the 20 wells. After the completion of the coupling, the block was filled with DMF up to 1 cm above the top of the wells and inverted, and the resin was mixed vigorously by agitation for 30 min in the mixing chamber. The block was again inverted, evenly distributing the resins into the wells for washing and removal of the Fmoc group. This procedure was repeated for the incorporation of all the randomized positions. After the randomized positions, the Fmoc-k(Boc) and Fmoc-K(Dnp) were incorporated. The side chain protecting groups were removed by treatment with a mixture of TFA/thioanisole/ethanedithiol/water (87:5:5:3) for 8 h. The resin was washed with 95% acetic acid (4×), DMF (4×), 5% DIPEA in DMF (3×), DMF (3×), and DCM (6×) and dried in vacuum.

Enzymes. Heterologous expression, purification, and active site titration of human cathepsin S were performed as previously described.[36] Recombinant NS2B-NS3 protease dengue 2 virus, strain NGC (CF40glyNS3pro], was obtained as reported.[37] β-trypsin was purified as described elsewhere[38] from a double-crystallized bovine trypsin from Biobras Co. (Montes Claros, Minas Gerais, Brazil), treated in advance with TPCK and the operational molarities were determined by active site titration.[39]

Solid-Phase Library Screening. For all assays, the library beads were washed with water (3×) and the assay buffer (3×) before the addition of the enzyme. The reactions were stopped by dilution with 3 M HCl, and the mixtures were washed thoroughly. The beads were transferred to a glass dish and inspected by fluorescence microscopy (Stereomicroscope Stemi−Zeiss), and the fluorescent beads were collected and transferred to a TFA-treated cartridge filter for on-resin sequence analysis. The amino acid sequence and cleavage point were determined by Edman degradation using the protein sequencer PPSQ/23, Shimadzu Co. The enzymes were assayed as follows: trypsin 20 mg of resin in 0.1 M Tris HCl, 10 mM $CaCl_2$ pH 8.0, 5 μM TPCK at 25 °C for 15 h with 4.2 nM of enzyme; chymotrypsin 20 mg of resin in 0.1 M Tris HCl, 10 mM $CaCl_2$ pH 8.0, 5 μM TLCK at 25 °C for 20 h with 4.4 nM of enzyme; cathepsin S 50 mg of resin, in 50 mM $NaPO_4$, 2.5 mM EDTA, pH 6.5, 25 °C for 7 h with 25 nM of enzyme (cathepsin S was preactivated with 10 mM dithioerytritol); NS3 protease dengue 2 virus 50 mg of resin in 50 mM Tris, 10 mM NaCl, 20% glycerol, pH 9.0, at 37 °C for 7 h with 30 nM of enzyme.

Kinetics of Hydrolysis by Chymotrypsin of the Peptides Attached to the Resin. The peptides bound to resin were incubated in 0.1M Tris HCl, 10 mM $CaCl_2$, pH 8.0, 5 μM TLCK, and 4.4 nM chymotrypsin. Samples were colleted every minute in the first 15 min and then after every 15 min. The samples collected were diluted with equal volume of 3 M HCl, and the released peptide was quantified by HPLC. A SPD-10AV Shimadzu UV−vis detector was used, coupled to an Ultrasphere C-18 column that was eluted with solvent system A1 (H_3PO_4/H_2O, 1:1000) and B1 (ACN/H_2O/H_3PO_4, 900:100:1) at a 1.0 mL/min flow rate. The hydrolysis products containing K(Dnp) were monitored and quantified at 365 nm using an authentic synthetic fragment. The same experiments were done in presence of 0.01% Tween.

Kinetics of Hydrolysis of the Peptides in Solution. The reactions were carried out for chymotrypsin at 37 °C in 0.1 M Tris HCl, 10 mM $CaCl_2$, 5 μM TPCK, pH 8.0. For trypsin, the same conditions as for chymotrypsin were used but in presence of 5 μM TLCK. Cathepsin S was preactivated with 10 mM dithioerytritol in 50 mM sodium phosphate, 2.5 mM EDTA, pH 6.5 for 5 min before the addition of the substrates. The hydrolysis was followed by measurement of the fluorescence intensity increase of Abz (λ_{EX} = 320 nm, λ_{EM} = 420 nm) in a Shimadzu RF-1501 spectrofluorometer. The concentrated stock solutions of the substrates (1 mg/mL, in H_2O/DMF 1:1) were diluted in water, and the final concentrations of the substrates were calculated from the absorption of 2,4-dinitrophenyl group (Dnp) (molar extinction coefficient at 365 nm was 17,300 $M^{-1}cm^{-1}$). Fluorescence variations were converted into amount of hydrolyzed substrates by standard curves obtained by the fluorescence measurement of well-defined concentrations of each substrate after complete hydrolysis. A 1 cm path length cuvette containing 1 mL of the substrate solution was placed in the thermostatic cell compartment. The enzyme solution was added, and the increase of fluorescence was continuously recorded. The slope was converted into moles of substrate hydrolysis per second, and the parameters were calculated according to Wilkinson.[40] Kinetic parameters K_m and k_{cat} with their respective standard errors were calculated by the Michaelis−Menten equation using Grafit software (Erithacus Software, Horley, Surrey, U.K.). For peptides hydrolyzed at more than one site, the apparent k_{cat}/K_m values were also determined under pseudo-first-order conditions (where $[S] << K_m$) and performed under two different substrate concentrations. Errors were 10% or less for each of the obtained kinetic parameters.

Abbreviations

NS, nonstructural; Q-EDDP, (glutamyl-*N*-[ethylenediamine] 2,4-dinitrophenyl); HOBt, *N*-hydroxybenzotriazole; TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluorborate; NMM, *N*-methyl-morpholine; (PMC), 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Trt, trityl; TPCK, tosyl phenylalanyl chloromethyl ketone; TLCK, tosyl lysil chloromethyl ketone

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