Exploring the subsite-structure of vimelysin and thermolysin using FRETS-libraries

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Abstract **Vimelysin is a metalloproteinase with high activity at low temperature and an unusual resistance to organic solvents. Substrate specificities of vimelysin and thermolysin were examined using FRETS-libraries, revealing a significant difference at the P3′ position: vimelysin preferred acidic amino acid residues, whereas thermolysin preferred basic residues. Homology modeling of vimelysin suggests that oppositely charged residues in the S3′ subsites (R215 in vimelysin and D213 in thermolysin) may be responsible for this specificity difference. This hypothesis was confirmed by examining the R215D mutant of vimelysin, which showed a substrate specificity profile intermediate between thermolysin and vimelysin.**

Keywords: Metallopeptidase; Thermolysin; Vimelysin; FRETS; Subsite-structure

1. Introduction

Vimelysin, a metalloproteinase from *Vibrio* sp. T1800 discovered by K. Oda et al. in 1996, is a unique enzyme characterized by its high activity at low temperature and its resistance to organic solvents [1–4]. Recently, we cloned and sequenced a gene encoding vimelysin (ORF: 1821 bp) [5]. Vimelysin is produced as a precursor comprising an amino terminal pre–pro region (195 amino acid residues), a mature region (311 a.a., MW; 34000) and a carboxy-terminal pro region (101 a.a.). The primary structure of vimelysin has the consensus catalytic site and metal-binding site motifs (HEXXH–E–H) belonging to the thermolysin family of metalloproteinases. The amounts of sequence identity between vimelysin and vibriolysin (*Vibrio proteolyticus*), Pseudolysin (*Pseudomonas aeruginosa*), and thermolysin (*Bacillus thermoproteolyticus*) are 81%, 57%, and 35%, respectively. An organic solvent resistant mutant of vimelysin, N123D, has also been constructed [5].

The substrate specificity of thermolysin and related enzymes has been thoroughly examined through many structural and kinetic studies [6–9]. The P1 position (immediately N-terminal to the cleavage site, according to the nomenclature of Schechter and Berger) and P1′ positions are the most important for substrate specificity: Phe is strongly preferred at the P1 position, and Leu or Phe at the P1′ position. In vibriolysin from *V. proteolyticus*, Phe is highly preferred for the P1′ position, and bulky amino acids at the P1 or P2 positions [10]. The specificity constants (k_{cat}/K_m) of vimelysin for *N*-[3-(2-furyl)acryloyl] (FA)-Gly–Leu-NH_2 (FAGLA) and for FA-Gly-Phe-NH_2 have been shown to be 0.13×10^5 and $2.07 \times 10^5\ M^{-1}\ s^{-1}$, respectively. The 15.9-fold higher k_{cat}/K_m value for the second substrate suggests that vimelysin shows a strong preference for Phe over Leu at the P1′ position [2].

In the current study, the substrate specificities of vimelysin and thermolysin were examined using three kinds of fluorescence resonance energy transfer substrate (FRETS)-combinatorial libraries. Substrate preferences at the P3, P2, P1, P1′, and P2′ positions were similar between vimelysin and thermolysin. In contrast, the P3′ preference was significantly different. Whereas vimelysin prefers neutral or acidic amino acids at the P3′ position, thermolysin shows a preference for basic or neutral amino acids. This intriguing difference in substrate specificity can be rationalized by comparing a three-dimensional homology model of vimelysin with the crystal structure of thermolysin: Arg 215 in vimelysin confers positive charge to the S3′ specificity pocket, whereas Asp 213 in thermolysin confers negative charge to the peptide-binding pocket, thus favoring interactions with positively charged side chains at the P3′ position of peptide substrates. To assess the importance of Arg 215 towards P3′ binding specificity in vimelysin, the substrate specificity of the R215D mutant of vimelysin was investigated. This single mutation altered the enzyme's P3′ substrate specificity away from wild-type vimelysin and towards a preference for basic residues, as seen in thermolysin. These experiments indicate that the difference in charged residues in the S3′ subsite of vimelysin and thermolysin is a major determinant of substrate specificity at the P3′ position.

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Abbreviations: FRETS, fluorescence resonance energy transfer substrate; Bz, Benzoyl; Nma, 2-(*N*-methylamino)benzoyl; D-A_2pr, 2,3-diamino propionic acid; LC-MS, Liquid chromatography mass spectrometer

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2. Materials and methods

2.1. Materials

Escherichia coli JM109 [*e14-(mcrA) recA1 endA1 gyrA96 thi-1 hsdR17*(r_k^-, m_k^+) *supE44 relA1*(*lac-proAB*) (F′*traD36 proAB lacIZ*^{q} M15)] was used as a host. pKK223-3-VLN [5] was used as a template for construction of R215D mutant of vimelysin. Restriction enzymes, *Pyrobest* DNA polymerase and DNA Ligation Kit ver. 2 were purchased from Takara Bio Co. Ltd. (Shiga, Japan). BigDye Terminator v1.1 Cycle Sequencing Kit was used for DNA sequencing (Applied Biosystems, California, USA). FAGLA (FA-Gly-Leu-NH_2) was purchased from Bachem Co. Ltd. (Bubendorf, Switzerland). FRETS-25Xaa, FRETS-25F, FRETS-36mix, and MOCAc-PLGL-A_2pr-(Dnp)-AR-NH_2 were a kind gift from Peptide Institute Inc. (Osaka, Japan). Determination of N-terminal amino acid sequence was performed by Shimazu Co. Ltd. (Kyoto, Japan).

2.2. DNA manipulations

General techniques of DNA manipulation were according to the method of Sambrook et al. [11].

2.2.1. Construction of R215D mutant of vimelysin. The R215D mutant of vimelysin was constructed by using the technique of overlap extension [12]. The PCR fragments were generated with primers VSF5 (5′-ACGTGGATTGGGTGGTCGGTAGC-3′) and 215R (5′-GGCATGATCAATCGAGTCACCATCTTTTGAAGGTTG-3′; mutation site is underlined), 215F (5′-CAACCTTCAAAAGATGGTGACTCGATTGATCATGC-3′) and VSR2 (5′-GGCATCTTCAACATCG GCAACC-3′). PCR fragments were generated using *Pyrobest* DNA polymerase and pKK223-3-VLN as a template. Both PCR fragments were purified by agarose gel electrophoresis, mixed and used as a template for the next PCR. A reaction mixture was prepared without primers and then DNA was denatured, annealed, and cooled down gradually and incubated at 72 °C for extension. Then after addition of VSF5 and VSR2 primers, PCR fragment was generated using *Pyrobest* DNA polymerase. The mutated DNA fragment was purified by phenol/chloroform/isoamyl alcohol extraction, ethanol precipitation, and subjected to digestion by *Eco*RV and *Bgl*II. The digested DNA fragment was further purified by agarose gel electrophoresis and the mutated DNA fragment was used to replace with wild-type vimelysin gene (*Eco*RV/*Bgl*II) in pKK223-3-VLN to construct R215D expression plasmid. The mutated site was confirmed by DNA sequencing. Expression and purification of the R215D mutant of vimelysin in *E. coli* was performed as described previously [5].

2.3. Analysis of enzyme properties

Enzymes were characterized by circular dichroism spectroscopy and N-terminal amino acid sequencing using the same methods described previously [5]. Enzyme kinetics were carried out using FAGLA (FA-Gly-Leu-NH_2) and MOCAc-PLGL-A_2pr (Dnp)-AR-NH_2 as substrates [5].

2.4. Substrate specificity

Analyses of substrate specificities using FRETS-libraries were performed as described previously [13–15]. Vimelysin, vimelysin R215D, and thermolysin were dissolved in buffer A (100 mM Tris–HCl, pH 7.5, containing 10 mM $CaCl_2$) and diluted to concentrations of 20 and 2 μg/ml, respectively.

2.4.1. Preference at the P1 position. Each of the 19 FRETS-25Xaa derivatives (Xaa = 19 kinds of amino acids) was dissolved in DMSO at a concentration of 1 mM. Calibration curves were prepared using FRETS-standard mixture (FRETS-25STD1 and FRETS-25STD2). For the reaction, a 10 μM solution of each FRETS-25Xaa (190 μl) was mixed with enzyme (20 ng/10 μl) and was reacted at room temperature. The intensity of the resulting fluorescence was measured using a microtiter plate reader.

2.4.2. Preference at the P2 and P3 positions. Calibration curves were prepared as follows: to a 100 μM solution of FRETS-25F, variable amounts of a 100 μM solution of FRETS-standard mixture were added to achieve a final concentration of 0%, 7.5% and 15% each and the fluorescence intensity was measured using a Hitachi 650-10S fluorescence-spectrometer (E_x = 340 nm, E_m = 440 nm). A control solution was made as follows: to the 100 μM solution of FRETS-25F (1 ml), 100 μl of reaction-stop solution (100 mM EDTA) was added, and then the solution was subjected to ODS-HPLC analysis. The sample (20 μl) was loaded and eluted using a gradient of CH_3CN concentration from 10% to 40% in the 0.05% TFA solution for 55 min and then 40% of CH_3CN solution for 5 min. The elution was monitored by fluorescence (E_x = 340 nm, E_m = 440 nm) and ultraviolet absorption (220 and 400 nm). For the reaction, 100 μM solution of FRETS-25F (1 ml) was put into a cuvette and incubated for 4 min at 37 °C, and then mixed with enzyme solution. The hydrolysis products were subjected to ODS-HPLC analysis. The amount of hydrolysis of the substrate was assumed to be proportional to the area of the fluorescence peak. Mass analysis was also carried out for the hydrolysis products using LC-ESIMS.

2.4.3. Preferences at the P1′, P2′ and P3′ positions. Preferences at the P′ sides were analyzed by use of the FRETS-36 mix as substrate under the same conditions used for FRETS-25F.

2.5. Homology modeling

Three-dimensional homology models of vimelysin were constructed using Modeller (v. 6.2) [16]. By using the deduced amino acid sequence of vimelysin (nucleotide sequence data accession number in the DDBJ/EMBL/GenBank databases: AB080995), the homology models of vimelysin were computed and analyzed (PDB code 2A4I). The crystal structures of pseudolysin (PDB code 1U4G [17]: in complex with *N*-(1-carboxy-3-phenylpropyl) phenylalanyl-α-asparagine) and thermolysin (PDB code 4TMN [18]: in complex with carbobenzoxy phenylalanine phosphinyl leucyl alanine) were used as templates for modeling.

3. Results and discussion

3.1. Substrate specificity of vimelysin and thermolysin using FRETS-25Xaa, FRETS-25F and FRETS-36 mix

3.1.1. FRETS-25Xaa. FRETS-25Xaa contains a highly fluorescent 2-(*N*-methylamino)benzoyl (Nma) group linked to the side chain of the amino-terminal D-2,3-diamino propionic acid (D-A_2pr) residue, which is efficiently quenched by a 2,4-dinitrophenyl (Dnp) group linked to the ε amino function of Lys. The chemical structure of FRETS-25Xaa is as follows: D-A_2pr (Nma) G–Zaa–Yaa–Xaa *AFPK (Dnp)–D–R–D–R, where an asterisk indicates the scissile site. Xaa represents the fixed position where each of the 19 natural amino acids excluding Cys was incorporated. A mixture of five amino acids (P, Y, K, I, and D) was incorporated at the Yaa position along with a mixture of five amino acids (F, A, V, E, and R) at the Zaa position for each fixed Xaa. This provides a peptide mixture with 25 combinations of each Xaa series, resulting in a combinatorial library with a total of 475 peptide substrates in 19 separate pools. Vimelysin cleaved the FRETS-25Xaa having Phe, Tyr, Met, and Thr at the Xaa position in decreasing order of effectiveness (Fig. 1). For thermolysin, the order of substrate preferences was Phe, Leu, Met, and Tyr. Both enzymes cleaved most effectively the FRETS-25Xaa having Phe at the P1 position. This result is in good agreement with previously reported studies on substrate specificity using various other assays [2].

3.1.2. FRETS-25F. The P2 and P3 preferences were analyzed using the FRETS-25F library. Both vimelysin and thermolysin cleaved the substrate, D-A_2pr (Nma) G–Zaa [F/A/V/E/R]–Yaa [P/Y/K/I/D]–Xaa[F]–*AFPK (Dnp)–D–R–D–R at the * position. The hydrolysis products (amino terminal side) are summarized in Fig. 2. Both enzymes preferred Lys and Tyr at the P2 position and Phe and Val at the P3 position.

3.1.3. FRETS-36 mix. In addition, preferences at the P′ positions were analyzed for both enzymes. Both enzymes