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Involvement of Val 315 located in the C-terminal region of thermolysin in its expression in *Escherichia coli* and its thermal stability

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ABSTRACT

Thermolysin is a thermophilic and halophilic zinc metalloproteinase that consists of β-rich N-terminal (residues 1–157) and α-rich C-terminal (residues 158–316) domains. Expression of thermolysin variants truncated from the C-terminus was examined in *E. coli* culture. The C-terminal Lys316 residue was not significant in the expression, but Val315 was critical. Variants in which Val315 was substituted with fourteen amino acids were prepared. The variants substituted with hydrophobic amino acids such as Leu and Ile were almost the same as wild-type thermolysin (WT) in the expression amount, α-helix content, and stability. Variants with charged (Asp, Glu, Lys, and Arg), bulky (Trp), or small (Gly) amino acids were lower in these characteristics than WT. All variants exhibited considerably high activities (50–100% of WT) in hydrolyzing protein and peptide substrates. The expression amount, helix content, and stability of variants showed good correlation with hydropathy indexes of the amino acids substituted for Val315. Crystallographic study of thermolysin has indicated that V315 is a member of the C-terminal hydrophobic cluster. The results obtained in the present study indicate that stabilization of the cluster increases thermolysin stability and that the variants with higher stability are expressed more in the culture. Although thermolysin activity was not severely affected by the variation at position 315, the stability and specificity were modified significantly, suggesting the long-range interaction between the C-terminal region and active site.

1. Introduction

Thermolysin [EC 3.4.24.27] is a thermophilic and halophilic neutral metalloproteinase produced in the culture broth of *Bacillus thermoproteolyticus* [1–3]. It has been widely utilized to the synthesis of peptides such as a precursor of an artificial sweetener aspartame [2,3], and thus is one of the most representative industrial enzymes.

It requires one zinc ion essential for enzyme activity and four calcium ions for structural stability [4–6]. The molecular mass is 34.6 kDa based on the amino acid composition (316 amino acids) plus one zinc and four calcium atoms [3]. It is a typical α + β protein [6] composed of the β-structure-rich N-terminal (residues 1–134) and α-structure-rich C-terminal (residues 135–316) domains. The majority of zinc metalloproteinases have the zinc-binding HEXXH motif. Thermolysin has the consensus motif, $H^{142}ELTH^{146}$, and His142, Glu143, and His146 are catalytically essential [2,7]. Its high-resolution crystal structures are available [7]. Many variants of thermolysin and thermolysin-like proteases (TLPs) with enhanced catalytic activity and stability have been obtained using site-directed mutagenesis [8–15]. A TLP-ste variant, which has 4 times higher activity than the wild-type enzyme, was constructed by the combined mutations of its four residues Asn116, Gln119, Asp150, and Gln225 [13], and the enzyme with enhanced stability was constructed by introducing a disulfide bond between positions 8 and 60 (the numbering of amino acid sequence of thermolysin was adopted for TLP-ste) [14,15]. We introduced mutation to amino acid residues whose C_α atoms locate within 1.2 nm from the catalytic zinc of thermolysin. The variants with mutation of Asp150, Ile168, and Asn227 give remarkable stabilization [9,10]. It is also stabilized by mutating Leu155 located at the autodegradation site [16]. Combination of the mutation of Ser65 to Pro and that of both Gly8 and Asn60 to Cys for introducing a disulfide bond as well as the case of TLP-ste [14] leads further stabilization [10,11]. Extensive combinations of effective mutations are more effective for increasing the activity and/or stability [9]. For example, activity of a variant in which Leu144 is converted to Ser, Asp150 to Glu, and Ser53 to Asp is 10 times higher than that of wild-type thermolysin (WT) and the inactivation rate at 80 °C is decreased to 60% of that of WT [9]. The stability of thermolysin is increased with stabilizing the Ca^{2+} ion (Ca3) which is located in the N-terminal domain [17,18].

It has been shown that the stability of the N-terminal domain (or N-domain) is lower than that of C-terminal domain (C-domain); thus the stabilization of the N-domain rather than of the C-domain has been considered more effective for stabilizing thermolysin [19]. The stabilization of the C-domain has been also effective for stabilizing variants

Abbreviations: CBB, Coomassie Brilliant Blue; CC, Correlation coefficient; CD, Circular dichroism; DA, Degree of activation; EA, Expression amount; FAGLA, *N*-[3-(2-Fury) acryloyl]-glycyl-L-leucine amide; HC, α-helix content; PU, Proteolytic unit; REA, Relative expression amount; RHC, Relative α-helix content; WT, Wild-type thermolysin

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in which N-domain is already enough stabilized [19]. Accordingly, stabilization of the C-domain is still useful for further stabilization of thermolysin. Previous studies on TLPs have shown that mutation of the C-terminus may indeed affect the stability of this type of proteases [20]. This suggests a possibility that some amino acid residues might have a significant role in the stability of the C-domain and of the total structure of thermolysin. Stabilized mutants of TLPs were predicted using computational techniques and produced successfully [21].

In the present study, we aimed to evaluate the effects of the C-terminal region of thermolysin on its activity and stability. It is known that proteins mutated to have lower stability have tendency to be easily digested in the host *Escherichia coli* cells [22] and we intended to testify this empirical relationship in the present study. We describe the effect of the truncation from the C-terminal end of thermolysin on its expression in the culture supernatant of the transformed *E. coli* cells and that the penultimate amino acid residue, Val 315, is important for the expression of thermolysin. The expression amount (EA) of the variants is in good agreement with the order of the hydropathy index of the amino acid residue incorporated in position 315. Hydrophobic amino acids are favored at position 315 for high EA of the variant, which exhibits also high stability and activity. These findings may provide some insights into the molecular mechanism for the folding of thermolysin and techniques to design recombinant enzymes with enough EA.

2. Materials and methods

2.1. Materials

Casein (Lot PEH5596) was purchased from Wako Pure Chemical (Osaka, Japan). *N*-[3-(2-Fury)acryloyl]-glycyl-L-leucine amide (FAGLA, Lot 111K1764) was from Sigma (St. Louis, MO). The concentration of FAGLA was determined spectrophotometrically using the molar absorption coefficient at 345 nm, $\varepsilon_{345} = 766\ M^{-1}\ cm^{-1}$ [3,23].

2.2. Expression and purification of thermolysin variants

Expression and purification of thermolysin variants were performed according to the method described previously [24] using *E. coli* K12 JM109 as host. Expression plasmids for the variants were constructed by polymerase chain reaction using primers listed in Table 1 and pTMP1 as a template. Site-directed mutagenesis and production of the variants were performed as described previously [9,24,25]. Briefly, the seed culture (5 ml) of the transformed JM109 cells was diluted 100 times with 500 ml of L broth in a 1-l flask and incubated at 37 °C for 48 h, with 0.1% (w/v) anti-foam A (Sigma) and vigorous aeration. At the end of the culture, a 500-ml supernatant was obtained. The cell number reached the maximum at around 24 h from the start of cultivation and decreased after this gradually. The casein-hydrolysis activity increased with the cultivation time and reached the maximum at around 36 h and this activity level was almost kept at 48 and 72 h with a slight increase [12]. The 48-h cultivation was confirmed to be optimal for expression of thermolysin variants before starting the present study.

Table 1
Nucleic acid sequences of primers used for constructing thermolysin variants.

Thermolysin variants	Primer sequence
All truncated variants (forward)	5′-TAAAGTGGTATCTCATCAGTGGGG-3′
Δ316 (reverse)	5′-CACCCCTACCGCATCAAAG-3′
Δ315–316 (reverse)	5′-CCCTACCGCATCAAAGGCC-3′
Δ314–316 (reverse)	5′-TACCGCATCAAAGGCCTGC-3′
Δ313–316 (reverse)	5′-CGCATCAAAGGCCTGCTTC-3′
Δ298–316 (reverse)	5′-ACCGTACAAGTCAGTGGCTGATTG-3′
V315G (forward)	5′-GATGCGGTAGGGGGGAAATAAAGTGG-3′
V315A (forward)	5′-GATGCGGTAGGGGCGAAATAAAGTGG-3′
V315L (forward)	5′-GATGCGGTAGGGCTGAAATAAAGTGG-3′
V315I (forward)	5′-GATGCGGTAGGGATTAAATAAAGTGG-3′
V315F (forward)	5′-GATGCGGTAGGGTTCAAATAAAGTGG-3′
V315Y (forward)	5′-GATGCGGTAGGGTATAAATAAAGTGG-3′
V315W (forward)	5′-GATGCGGTAGGGTGGAAATAAAGTGG-3′
V315S (forward)	5′-GATGCGGTAGGGTCGAAATAAAGTGG-3′
V315T (forward)	5′-GATGCGGTAGGGACGAAATAAAGTGG-3′
V315Q (forward)	5′-GATGCGGTAGGGCAGAAATAAAGTGG-3′
V315D (forward)	5′-GATGCGGTAGGGGACAAATAAAGTGG-3′
V315E (forward)	5′-GATGCGGTAGGGGAGAAATAAAGTGG-3′
V315R (forward)	5′-GATGCGGTAGGGCGTAAATAAAGTGG-3′
V315K (forward)	5′-GATGCGGTAGGGAAGAAATAAAGTGG-3′

"Forward" or "reverse" in parentheses shows that the primer was used as a forward primer or as a reverse primer, respectively. Underlined nucleic acid sequences in primers for Val315 variants indicate mutation sites. Reverse primers for Val315 variants (not shown in this table) have nucleic acid sequence complementary to respective forward primers.

For the purification of thermolysin variants, 3 l of the culture supernatants of *E. coli* transformants with low protein expression (Δ315–316, Δ314–316, Δ313–316, Δ312–316, Δ298–316, V315G, V315W, V315D, V315E, V315R, and V315K) obtained by cultivation for 72 h were used, while 0.5 l were used for the other transformants (see Section 3.2). Thermolysin variants were purified to homogeneity from the culture supernatant with hydrophobic-interaction chromatography followed by affinity chromatography with a column of glycyl-D-phenylalanine (Gly-D-Phe) coupled to Sepharose 4B resin [9]. One-step purification of TLPs using Bacitracin-silica has been applied [26]. However, we have preferred the Gly-D-Phe gels because of pharmaceutical effects of Bacitracin considering potential application of thermolysin to food industry. Prior to kinetic measurements, the preparations were desalted using pre-packed PD-10 gel filtration columns (Amersham, Uppsala, Sweden) in 40 mM Tris–HCl (pH 7.5) (designated as buffer B) added with 10 mM $CaCl_2$ (which is designated as buffer B*) and stored at 4 °C.

2.3. Sodium dodecyl sulfate-polyacryl amide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in a 12.5% polyacrylamide gel under reducing conditions with a constant current of 40 mA for 40 min [27]. Samples were reduced by treatment with 2.5% 2-mercaptoethanol at 100 °C for 10 min. Proteins were stained with Coomassie Brilliant Blue (CBB) R-250. The protein molecular-mass marker kit was from Takara (Otsu, Japan).

2.4. Hydrolysis of casein

The casein-hydrolysis activity of thermolysin was determined according to the method described previously [2,11]. The thermolysin solution (187.5 μl) was added to 562.5 μl of 1.33% (*w*/*v*) casein in buffer B, and incubated at 25 °C for 30 min. The reaction was stopped by adding 750 μl of a solution containing 0.11 M trichloroacetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid. After 30-min incubation at 25 °C, the reaction mixture was filtered through Whatman No. 2 filter paper, and the absorbance at 275 nm (A_{275}) was measured. One proteolytic unit (PU) of activity is defined as the amount of enzyme activity needed to liberate a quantity of acid soluble peptides corresponding to an increase in A_{275} of 0.0074 $cm^{-1}\ min^{-1}$ (A_{275} of 1 μg of tyrosine per min).

2.5. Hydrolysis of FAGLA

The thermolysin-catalyzed hydrolysis of FAGLA was measured by following the decrease in absorbance (A_{345}) at 345 nm in buffer B* at 25 °C [3,23]. The amount of FAGLA hydrolyzed was evaluated using the molar absorption difference due to hydrolysis, $\Delta\varepsilon_{345} = -310\ M^{-1}\ cm^{-1}$. The hydrolysis was carried out under pseudo-first-order conditions, where the substrate concentration is much lower than the Michaelis constant (K_m) (>30 mM) [3]. Under the conditions, the enzyme activity was evaluated by the specificity constant (k_{cat}/K_m).