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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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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.

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.

Keywords: C-terminal region; enzyme expression; enzyme stability; hydropathy; site-directed mutagenesis; thermolysin.

1. Introduction

Thermolysin [EC 3.4.24.27] is a thermophilic and halophilic neutral metalloproteinase produced in the culture broth of *Bacillus thermoproteolyticus* [1, 2, 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^{o}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 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 hydrophathy 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, ε_{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-liter flask and incubated at 37^{o}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.

For the purification of thermolysin variants, three liters 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 liters 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^{o}C.

Table 1

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).

168 *2.5. Hydrolysis of FAGLA.*

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

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178 *2.6. Far-UV CD spectra.*

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180 The CD spectra were measured using 0.2-cm cell with a Jasco J-820
181 spectropolarimeter (Tokyo, Japan) equipped with a Peltier system of cell temperature
182 control. The measurement conditions were: spectral range, 200-250 nm; sensitivity, 100
183 mdeg; resolution, 0.2 nm; response time, 4 s; scan speed, 20 nm min^{-1}; the number of
184 accumulations, 4; and the protein concentration, 1.0 μM in buffer B* [16]. The α-helix
185 content was calculated from the mean-residue molar ellipticity at 222 nm ($[\theta]_{222}$ (deg
186 $cm^2\ dmol^{-1}$)) [28].

187

188 *2.7. Thermal denaturation of thermolysin.*

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190 Thermolysin (2.5 μM) in buffer B* was incubated at 25°C for 5 min. Then the
191 solution (400 μl) was transferred to a 0.2-cm quartz cell, and mineral oil (50 μl) was

added to avoid evaporation. Thermal denaturation was examined by monitoring θ_{222} with increasing cell temperature from 60 to 100°C at a rate of 0.5°C min^{-1}. The fraction unfolded (F_u) was determined after normalizing θ_{222} of native and denatured proteins between 0 and 1, according to eq. 1.

$$F_u = (A_o - A_N) / (A_D - A_N) \quad (1)$$

where A_o is the observed θ_{222} of thermolysin at a temperature and A_N and A_D are the θ_{222} values of native and denatured proteins, respectively. The denaturation process is irreversible and is accompanied with autolysis [19, 20]. The temperature at which F_u is 0.5 is defined as the observed melting temperature (the temperature at which 50% of the thermolysin molecules is denatured), $T_{m, app}$.

2.8. Thermal inactivation of thermolysin.

Thermolysin (2.5 μM) in buffer B* was incubated at a temperature ranging from 60 to 75°C for a specified time (2-32 min). Then, it was cooled at 25°C for 5 min and the FAGLA-hydrolysis activity was determined by the method described above. It is known that the thermal inactivation of thermolysin is irreversible and consists of only one step, the first-order rate constant, k_{obs}, of the inactivation was evaluated by plotting logarithm of the residual activity (k_{cat}/K_m) against the duration time of thermal treatment [19, 20]. The activation energy, E_a, for the inactivation was determined according to Arrhenius equation by plotting logarithm of k_{obs} against ($1/T$) where T is the temperature in Kelvin. Thermal inactivation of thermolysin was handled to obey the first-order reaction, eq. 2:

$$\ln([E]_t/[E]_o) = -k_{obs}\, t \qquad (2)$$

where $[E]_t$ and $[E]_o$ are the enzyme concentrations at incubation time (t) at temperature T and at time zero. The temperature (T_{50}) is defined as a temperature at which the enzyme loses 50% of the activity in the incubation for 30 min. Thus T_{50} is represented as a temperature at which the k_{obs} value is given as 3.85×10^{-4} s^{-1} when the enzyme is incubated for 30 min.

3. Results

3.1. Expression of truncated thermolysin variants.

A truncated thermolysin variant, Δ298–316, lacking the C-terminal α-helix (residue 298–316) was failed to be produced in the expression system used, suggesting that amino acid residues necessary for thermolysin expression might be contained in this α-helix. The transformants for the variants lacking Lys316, Val315–Lys316, Gly314–Lys316, and Val313–Lys316, designated as Δ316, Δ315–316, Δ314–316, and Δ313–316, respectively, were cultivated in *E. coli* at 37°C for 48 h. The 34.6-kDa protein bands corresponding to thermolysin were detected in SDS-PAGE for the supernatants of WT and Δ316 but not those of Δ315–316, Δ314–316, and Δ313–316 (Results not shown). The casein-hydrolysis activity in the supernatant of Δ316 was 126±1 PU/ml comparable to that of WT and not detected with Δ315–316, Δ314–316, and Δ313–316 (detection limit: 2 PU/ml). These results indicate that truncation of the

C-terminal Lys316 has no effect on the thermolysin expression but the penultimate residue Val315 is critical.

Table 2

3.2. Expression and purification of Val315 variants of thermolysin,

The expression of fourteen Val315 variants was examined (Fig. 1). The casein-hydrolysis activities observed in the culture supernatants of the variants varied but well correlated with the intensities of the 34.6-kDa protein bands corresponding to thermolysin variants. The variants were classified into four groups based on their casein-hydrolysis activities (PU/ml-supernatant) produced in the supernatants. As the volumes of the supernatants produced from all cultures for 48 h at 37^{o}C were almost the same as 500 ml, this classification is that based on the total casein-hydrolysis activities of the supernatants : Group A, showing 104-91% activity relative to that of WT, includes Δ316, V315L, and V315I; group B, showing 77-66%, includes V315A, V315T, and V315F; group C, showing 39-21%, includes V315S, V315Q, and V315Y; and group D, showing 8-2%, includes V315G, V315W, V315D, and V315E, V315K, and V315R (Table 2).

Fig. 1

All variants were purified to homogeneity as judged by SDS-PAGE (Fig. 2) and showed the molecular masses of 34.6 kDa. They are produced in *E. coli* culture

supernatants and purified in an intact form without any damage.

Fig. 2

3.3. Far-UV CD spectra of thermolysin variants.

CD spectra of WT and the variants were measured in buffer B* (pH 7.5) at 25^{o}C (Fig. 3) and the α-helix contents (HCs) were calculated from the $[\theta]_{222}$ values (Table 2). The HC of WT was 36%, consistent with that previously reported [16]. The HCs of the variants of group A, B, and C (classified in section *3.2*, Table 2) are 36-31%. Among them, the HCs (36-34%) of Δ316, V315L, V315Y, and V315T are in good agreement with that of WT, while those (32-31%) of V315A, V315I, V315F, and V315S are in reasonable agreement. On the other hand, the HCs of V315Q and the variants of group D are considerably low (29-22%). In other words, the relative α-helix contents (RHCs) of the variants in groups A-C and D are 100-86% and 81–61% to that of WT, respectively. This suggests that the degree of expression of the variants has some correlation with their secondary structures.

Fig. 3

3.4. Casein- and FAGLA- hydrolysis activities of thermolysin variants.

The Δ316, Δ315-316, and all Val315 variants showed casein- and FAGLA-hydrolysis activities at pH 7.5 and at 25^{o}C (Tables 2 and 3). All variants except

for V315W keep specific activities toward both substrates higher than 70% of those of WT. The activities of V315W are significantly low. The orders of the variants in the activities toward both substrates are almost the same. The relative activities of V315G for casein and FAGLA are 80% and 70% of those of WT. The substitution of Val315 with a charged residue decreased the activity in a complicated manner. The relative casein-hydrolysis activities of V315R and V315K were 70 and 50%, whereas those of V315D and V315E were 80 and 90%. The relative FAGLA-hydrolysis activities of both V315D and V315E were 70%, whereas those of V315R and V315K were 90 and 80%. By taking ratios of the relative activities for casein and FAGLA of the respective variants, namely by dividing the numerals in parentheses in the column of the casein-hydrolysis activity of purified variants (Table 2) by those in the column of the (k_{cat}/K_m) values at 0 M NaCl of the FAGLA-hydrolysis activity (Table 3), substrate specificity of the variants was evaluated. The order is: V315W (1.5) >> V315E (1.3) > V315G (1.2) > Δ315-316 = V315D = Δ316 (1.1) > WT = V315L = V315I = V315Y (1.0) > V315T = V315A = V315F = V315Q (0.9) > V315S = V315R (0.8) >> V315K (0.6). This order indicates that V315W, V315E, and V315G favor casein rather than FAGLA while V315K, V315R, and V315S favor FAGLA rather than casein. The characteristics of the residue incorporated at position 315, which is 2.2 nm far from the active site, are not so critical to the enzyme activity. However, some amino acids incorporated changed significantly the substrate specificity. This effect might be transferred through a long-range interaction from the C-terminal region to the active site.

Table 3

312

313 *3.5. Expression amounts of thermolysin variants.*

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315 The expression amount (EA) of the variant was defined as a weight (mg) of the
316 variant protein produced in a 500-ml culture supernatant during the cultivation of the
317 transformant at 37^{o}C for 48 h. It was calculated in the division of the total
318 casein-hydrolysis activity [proteolytic unit (PU)] contained in the supernatant by the
319 specific activity [PU/mg] of the variant purified from the supernatant (Table 2). The EA
320 (μg/ml) given in Table 2 is shown as the weight of the variant in one-ml of the
321 supernatant out of 500 ml. In the case of WT, the EA was 9.3±0.7 μg/ml, thus
322 indicating that the total amount of the WT protein produced in the culture must be 4.7±
323 0.4 mg. The relative EA or REA (as shown in the parentheses of the 3rd column in
324 Table 2) was defined as the ratio of the EA of the variant to that of WT.

325 The EAs of Δ316 and V315L were almost the same as that of WT, and that of
326 V315I was 20% higher than that of WT. Those three are the members of group A
327 (section *3.2*). V315F, V315A, and V315T, giving REAs of 90 to 80%, are the group B
328 members. V315Q, V315S, and V315Y, giving 40 to 20%, are the group C members.
329 V315G, V315K, V315R, V315W, V315D, and V315E, giving < 10%, are the group D
330 members. Grouping of the variants based on the EA is in good agreement with that
331 based on the casein-hydrolysis activity in the supernatants (section *3.2*).

332

333 *3.6. Effect of NaCl on the catalytic activities of thermolysin variants.*

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335 Thermolysin is remarkably activated by high concentrations (1-5 M) of

mono-valent salts such as LiCl, NaCl, KBr *etc.* and the activity increases in an exponential fashion with increasing salt concentration [3, 29, 30]. This activation is solely due to increase in k_{cat}. The effects of NaCl on the activity of the variants were examined (Table 3). The degree of activation (DA) is defined as the value obtained by dividing the FAGLA-hydrolysis activity in the presence of 4 M NaCl by that in the absence, and the value of WT was 17 [29]. The DAs of the variants were all within the range of 15–18, indicating that the modification at Val315 and truncation of the C-terminal residues examined here give substantially no effect on the salt-activation of thermolysin. Relationship between the salt-activation, and effects of anions and cations of salts on the structures of thermolysin and bulk water have been suggested [30, 31]. The present result suggests that Na^+ and/or Cl^- ions have no significant interaction with the C-terminal end of thermolysin. The hydrophobic interaction is stabilized with high concentration of salts, although electrostatic and hydrogen-bonding interactions are destabilized [32]. The variants in which Val315 was replaced with more hydrophobic residues like Leu, Ile, and Trp showed higher DAs than those with less hydrophobic ones. This suggests that the stability of the C-terminal hydrophobic cluster might be significant for the enzyme activity.

3.7. Thermal unfolding of thermolysin variants.

The effect of substituting Val315 with any other amino acid residues on its stability was examined by CD measurement. The CD signal at 222 nm ($[\theta]_{222}$), was continuously measured with increasing the cell temperature (Fig. 4) and the $T_{m,\,app}$ was determined for each variant (Table 2). The $T_{m,\,app}$ of WT was 88.8^{o}C being in good agreement with that

360 measured by differential scanning calorimetry [33] and the variants examined are all
361 destabilized in comparison with WT. The order of the variants in the $T_{m, app}$ values: WT
362 > (Δ316 = V315I) > (V315L = V315T = V315A) > V315F > V315S > V315Q > V315Y
363 > V315D > V315G > V315E > V315K > V315W > V315R > Δ315-Δ316. The effect of
364 the amino acid substitution was assessed by the difference ($\Delta T_{m, app}$) in the $T_{m, app}$ of the
365 variant from that of WT [namely, $\Delta T_{m, app}$ = ($T_{m, app}$ of the variant) – ($T_{m, app}$ of WT)].
366 The variants classified in group A (Table 2, section *3.2*) show relatively small $\Delta T_{m, app}$
367 values ranging from -2.2 to -1.0°C. The $\Delta T_{m, app}$ of Δ316 was -1.0°C and that of
368 Δ315-316 was -12.8°C, indicating that Δ316 is the most stable and Δ315-316 is the most
369 unstable among all examined. Deletion of the C-terminal Lys316 is not much effective
370 on the stability, although additional deletion of Val315 causes extensive damage,
371 suggesting that Val315 plays a critical role in thermolysin stability. This result is well
372 correspondent with that observed with *B. subtilis* neutral protease [20]. The $T_{m, app}$ of
373 Δ315-316 is similar to those of V315E, V315K, V315W, and V315R, showing that Glu,
374 Lys, Trp, and Arg residues at position 315 have no contribution in the stability. Good
375 correlation is observed between the $T_{m, app}$ values of the variants and their EAs (Table 2,
376 see Fig. 7C). The $\Delta T_{m, app}$ values of the variants in groups B, C, and D were in the
377 ranges of -3.5 to -2.2; -8.0 to -4.0; and -12.8 to -8.0, respectively, indicating that the
378 higher the EA of the variant is, the higher its stability is.
379
380 Fig. 4
381
382 *3.8. Thermal inactivation of thermolysin variants.*
383

Thermal inactivation of variants V315I, V315T, and V315R was examined by incubating at temperatures of 60, 65, 70, and 75°C. The semi-logarithmic plots of the remaining activity after thermal incubation against the incubation time gave the linear relationship, suggesting that all inactivation processes followed pseudo-first order kinetics (Fig. 5). The first-order rate constant k_{obs} (s^{-1}) values for thermal inactivation of WT, V315I, V315T, and V315R increased with increasing temperatures from 60 to 75°C, and those at 75°C were (0.87±0.10) x 10^{-3}, (0.80 ± 0.10) x 10^{-3}, (0.93±0.10) x 10^{-3}, and (1.98±0.10) x 10^{-3}, respectively. Linear relationship was observed in Arrhenius plot for k_{obs} to evaluate the activation energy (E_a) for thermal inactivation. The E_a values (kJ mol^{-1}) are as in the order: V315R (95 ± 3) > WT (66 ± 3) > V315I (60 ± 1) > V315T (60 ± 5). On the other hand, the T_{50} (°C) values of the variants were calculated and are in the order of WT (60.5 ± 0.1) = V315I (60.5 ± 0.7) > V315T (58.2 ±0.9) > V315R (57.5 ± 0.6). Interestingly, V315R has the largest E_a and smallest T_{50} values among the variants examined.

Fig. 5

4. Discussion

4.1. Relationship between the activity and stability of Val315 variants.

Group A variants show 120-100% in REAs and 95-87% in RHCs comparing with those of WT. Group B variants show 80-70% in REAs and 99-86% in RHCs, 40-20% in REAs and 95-76% in RHCs; and group D variants, 10% or less in REAs and 80-62% in

RHCs. The enzyme activities of the variants for both casein and FAGLA substrates decrease with decreasing REA and RHC and also decreasing the stability as given by $\Delta T_{m,\,app}$. It is noticeable that the enzyme activities are not much clearly distinguished between groups B and C, though the REA, RHC, and $\Delta T_{m,\,app}$ are distinctly different between them.

4.2. Important roles of the residue at position 315 in the expression of thermolysin.

The truncation of the C-terminal Lys316 as shown with the variant Δ316 has no effects on the EA, enzyme activities, $T_{m,\,app}$, and HC. This result is consistent with the crystal-structural data indicating that Lys316 projects outward from the C-terminal α-helix with no interactions with other residues. On the other hand, Val315 points toward the hydrophobic cluster formed by three α-helices together with Tyr217, Ile236, Phe281, Ser282, Arg285, Phe310, and Val313 (Fig. 6) [7]. TLPs from *B. stearothermophilus* and *B. cereus* bear Val315 and the C-terminal residues at position 316 of Tyr and Asn, respectively, as well as Lys of thermolysin, and those of *B. subtilis* and *B. amyloloquefacience* lack the residue at position 316 and Leu315 is the C-terminal end [20]. It seems that the residue at position 316 is not always essential for TLPs. Val315 of TLPs plays an important role in forming the hydrophobic cluster [34]. The thermostability of *B. subtilis* neutral protease was lowered by deleting Leu300 (correspondent to Val315 in thermolysin) or by replacing this by a polar amino acid, although the stability was increased upon replacing Leu300 by Phe [20]. The Val315 variants in which Val315 is replaced with hydrophobic amino acid residues exhibited the similar EAs to that of WT (Table 2). In contrast, those replaced with hydrophilic

residues, especially with charged ones, exhibited much lower EAs than that of WT.

Fig. 6

The hydropathy index is regarded to represent the combined character of hydrophobic and hydrophilic tendencies of the side chain [35], and thus, should be suitable for characterizing amino acid residues in the protein structure [29]. The REAs (%) of the Val315 variants (Table 2) were plotted against the hydropathy indexes of the amino acid residues introduced into position 315 (Fig. 7A). A positive linear correlation was observed with a correlation coefficient (CC) of 0.90 and the slope of 12, indicating that the REA increases by 12% as the increase in hydropathy of 1.0 unit.

Fig. 7

4.3. Important roles of the residue at position 315 in the secondary structure of thermolysin.

The α-helix content (HC) of WT is 36%, and those of the variants in groups A-C and D (Table 2, section *3.4*) are 36–32% and 30–25%, respectively. Thermolysin contains seven α-helix segments (residues 67–87, 137–152, 159–180, 225–246, 260–274, 282–296, and 301–313) [6]. Assuming that the HC of WT is given as the sum of the seven helix segments, the contribution of one segment to the HC might be approximately 5% in average. The difference in the HCs of the variants of groups A-C from WT are 0-4% and those of the variants of group D are 6-11%, suggesting that one

of the α-helices might be unfolded in the former variants, and one or two may be unfolded in the latter variants. As V315 is only manipulated to prepare variants, the unfolded α-helices might be those at the C-terminal region of thermolysin. It should be noteworthy that partially-unfolded variants maintain the steric structure avoiding catastrophic unfolding and exhibiting considerably high activities (Tables 2 and 3). The unfolding processes (Fig. 4) represent microscopic irregular changes including autolysis, and thus the processes might not obey a simple two-phase transition model. Especially, the process of V315W obviously has two inflection points, suggesting at least two steps in unfolding. The partial unfolding of the α-helices does not bring severe damages to the activity, and this is consistent with the suggestion that the partially-unfolded helices might be located at the C-terminal region far enough from the active site.

A possibility can be raised that a variant with low HC might be an equilibrium mixture of the extensively-unfolded forms and intact form of thermolysin, although this possibility might not be probable. The variants were purified homogeneously by affinity chromatography in which the affinity ligand (Gly-D-Phe) is designed so to bind with the substrate binding site and enzyme preparations thus purified might have enzyme activities as high as that of WT. If any extensively-unfolded form was present with the intact enzyme, the former might be degraded easily by autolysis and the resulting fragments might be observed in SDS-PAGE, though no such fragments were detected and the protein giving 34.6 kDa in SDS-PAGE did not disappear even in a long incubation of the variant solution.

4. 4. Important roles of the residue at position 315 in the thermostability of thermolysin.

If the unfolding of the α-helices of thermolysin was reversible, the unfolding was considered to obey two-state denaturation model, and the thermodynamic parameters, such as the changes in Gibbs energy, enthalpy, and enthalpy (ΔG^o, ΔH^o, and ΔS^o) under the standard conditions can be calculated. However, the reversibility was not observed in the CD signal at 222 nm and the thermolysin protein was degraded into small fragments when WT was cooled to 60°C after heated up to 100°C, and this observation is consistent with that previously reported [19, 21, 36, 37]. Therefore, thermal inactivation of thermolysin is given by the combined effects of unfolding and autolysis, and the preferential autolysis site was identified as Leu155-Ile156 [16]. Considerably-high enzyme activities of all variants suggest that the autolysis would be occurred in elevating temperature in the denaturation study (Fig. 4). Thus, the $T_{m,\ app}$ must be reflected by not only unfolding but also autolysis (Table 2) [19, 21]. As the enzyme activities of the variants are almost the same, the contribution of autolysis in $T_{m,\ app}$ should be almost the same with all variants, and so the variation in $T_{m,\ app}$ may be mainly connected with the stability of thermolysin, more precisely of the α-helices at the C-terminal region.

The plot of $T_{m,\ app}$ of the V315 variant against the hydrophathy index of the amino acid at position 315 gives a positive correlation with a CC of 0.84 (Fig. 7B). The slope of the plot is 1.1, indicating that $T_{m,\ app}$ increases by 1.1°C as the increase in hydropathy index of 1.0 unit. The order of WT, V315I, V315T, and V315R for T_{50} values is also in good agreement with that of hydropathy index of the amino acid introduced at position 315. It is considered that a hydrophobic cluster formed around V315 stabilizes the connection of two α-helix segments (residues 282-296 and 301-313) at the C-terminal region [7]. V315 interacts with F218 and I237 through the hydrophobic effect. F218 and

I237 also interact with hydrophobic A163 and A167, respectively. E166 which coordinates the zinc ion and is an important residue to express the enzyme activity is located between A163 and A167 on α-helix structure. Therefore, there is a possibility that the hydrophobic properties of the residues at 315 might influence the coordination geometry around the zinc ion and the enzyme activities.

4.5. Correlation between the expression amounts and $T_{m, app}$ values of Val315 variants.

A positive correlation was observed in the plot of the $T_{m, app}$ of the V315 variant against the REA with a CC of 0.94 (Fig. 7C). The slope of the plot is 0.094, indicating that the $T_{m, app}$ increases by 0.94°C as REA increases by 10%. The higher the stability of the variant is, the higher the amount of the variant produced in the culture supernatant is. It has been reported that proteins destabilized by mutagenesis are more susceptible to intracellular degradation [22]. The result in Fig. 7C suggests that the variant with $T_{m, app}$ of 78°C would not be produced in the *E. coli* culture and that increasing the stability is a key factor to produce higher amount of thermolysin using the *E. coli* system.

4.6. Roles of the residue at position 315 in casein- and FAGLA-hydrolytic activities of thermolysin.

The enzyme activities of the variant for casein and FAGLA were plotted against hydrophathy index of the amino acid incorporated into position 315 (data not shown). A slight correlation was observed showing that the activities of variants having hydrophobic amino acids at position 315 are higher than those having hydrophilic

amino acids. The amino acid at position 315 not only affects the stability of the C-terminal cluster and thus the gross structure of thermolysin but also affects the catalytic events executed at the active site.

Recombinant enzymes are used industrially for clinical diagnoses [38] and synthesis of intermediates of medicine and pesticide [39]. The EA of recombinant enzyme is an important issue because the cost of enzyme is fairly high and determines the cost of the products. Our results demonstrate that Val315, which locates surrounded by C-terminal α-helices, is important for the stability of thermolysin. Results of the single-amino-acid-substitution showed that there are good correlations among the thermostability ($T_{m, app}$) of thermolysin, stability of C-teminal α-helices, the EA, and the hydropathy index of the amino acid incorporated into position 315. The variants having hydrophobic residues at position 315 (such as WT, V315I, and V315L) show higher thermostability and HCs probably by stabilizing the C-terminal hydrophobic cluster and they give higher EAs in *E. coli* culture. On the other hand, the variants having hydrophilic, bulky, or small residues (such as V315R, V315E, V315W, and V315G) show lower thermostability and HCs probably by destabilizing the C-terminal hydrophobic cluster, and give the lower EAs. All variants exhibit considerably high enzyme activity. Thus, the stability of the gross structure and α-helix content are not directly related with the activity. The decrease in the HC observed with the low-stability variants from that with the high-stability variants might be due to destabilization of the C-terminal hydrophobic cluster. This suggests that stability in the C-terminal region has a significant effect on production of the variants in *E. coli* culture and that the variants with lower stability would be degraded during the folding and/or secretion processes. It is reminded that WT is the best as well as V315I and V315L in thermostability and EA

552 and even in enzyme activity, and that the Val residue is most optimally selected at
553 position 315 by nature.
554
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672 Figure Legends

673

674 Fig. 1. SDS-PAGE analysis of culture supernatants of *E. coli* transformed with
675 expression plasmids for Val315 variants of thermolysin. The supernatants of the *E. coli*
676 cultured in LB medium at 37^{o}C for 48 h were analyzed by SDS-PAGE. An arrow
677 labeled with TLN indicates the position of 34.6 kDa corresponding to the molecular
678 mass of thermolysin. Molecular-mass marker proteins are rabbit muscle phosphorylase
679 *b*, bovine serum albumin, hen egg-white ovalbumin, bovine carbonic anhydrase,
680 soybean trypsin inhibitor, and hen egg white lysozyme with the molecular sizes of 97.2,
681 66.4, 44.3, 29.0, 20.1, and 14.3 kDa, respectively. Panel A. Marker proteins (lanes 1 and
682 11); native thermolysin from *B. thermoproteolyticus* (lanes 2 and 10); the supernatants
683 of *E. coli* transformed with the expression plasmids for V315G, V315A, V315L, V315I,
684 V315F, V315Y, and V315W (lanes 3 to 9, respectively). Panel B. Marker proteins (lanes
685 1 and 12); native thermolysin (lanes 2 and 11); the supernatants of *E. coli* transformed
686 with the expression plasmids for V315K, V315R, V315D, V315E, V315N, V315Q,
687 V315S, and V315T (lanes 3 to 10, respectively).

688

689 Fig. 2. SDS-PAGE analysis of purified wild-type (WT) thermolysin and thermolysin
690 variants. One micro-gram each of WT and the variants was loaded onto the gel. An
691 arrow at the left of the gel indicates the position of 34.6 kDa. Marker proteins are as
692 described in Fig. 1. Panel A. Marker proteins (lanes 1 and 12); WT, Δ316, V315A,
693 V315L, V315I, V315F, V315Y, V315Q, V315S, and V315T (lanes 2 to 11, respectively).
694 Panel B. Marker proteins (lanes 1 and 10); WT, Δ315–316, V315G, V315W, V315D,
695 V315E, V315K, and V315R (lanes 2 to 9, respectively).

Fig. 3. CD spectra of WT thermolysin and thermolysin variants. CD spectra of WT and the variants (1 μM) in 40 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ (buffer B*) were recorded at 25°C. Panel A. WT, Δ316, V315A, V315L, V315I, V315F, V315Y, V315S, and V315T from the bottom to the top; and Panel B. WT, V315Q, Δ315–316, V315G, V315W, V315D, V315E, V315K, and V315R from the bottom to the top.

Fig. 4. Thermal denaturation of WT thermolysin and thermolysin variants. WT and the variants (2.5 μM) in buffer B* (pH 7.5) were heated from 60 to 100°C at a rate of 0.5°C/min, and the changes in $[\theta]_{222}$ were recorded continuously. The denaturation curves of WT and 16 variants were measured and those of WT, Δ315–316, V315G, V315F, V315W, and V315K were shown as representatives.

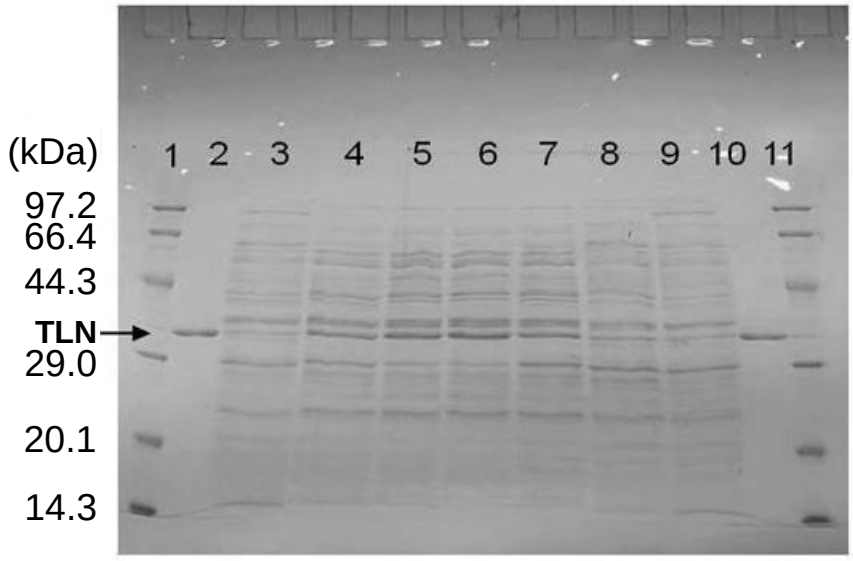
Fig. 5. Thermal denaturation of WT thermolysin and thermolysin variants at various temperatures. FAGLA-hydrolysis activities of WT and the variants were measured at 25°C after thermal incubation at 60, 65, 70, and 75°C (panels A-D, respectively) for specified time. The relative activity (k_{cat}/K_m) was expressed as the ratio of the activity observed after thermal incubation to that before the incubation. The natural logarithms of relative activities of WT and variants were plotted against incubation time at the specified temperature. The horizontal dotted line shows ln [Relative activity] given when the relative activity is 50%. Symbols: WT, ○; V315I, △; V315T, □; and V315R, ◇.

Fig. 6. Structute of thermolysin. The thermolysin structure is illustrated based on

Protein Data Bank PDB number 8TLN [7]. Panel A. Overall structure. Panel B. Close-up view of the C-terminal region.

Fig. 7. Correlation between the relative expression amounts (REAs) and the observed melting temperatures ($T_{m,\ app}$) of thermolysin variants and hydropathy indexes of the amino acid residues at position 315. Panel A. Correlation between the REAs and hydrophathy indexes. The REA (%) is the ratio of the amount of the variants expressed in the culture supernatants incubated at 37^{o}C for 48 h to that of WT (9.3 ± 0.7 mg/l-supernatant). The amino acid residue at position 315 is shown as a single-letter amino acid code. The data were fitted by the least-squares regression (LSR) to a linear line given by y = 12x + 50 and *r* (correlation coefficient) = 0.90. Panel B. Correlation between the $T_{m,\ app}$ values and hydrophathy indexes. The data were fitted by LSR to a linear line given by y = 1.1x + 83 and *r* (correlation coefficient) = 0.84. Panel C. Correlation between the $T_{m,\ app}$ values and their REAs. The data were fitted by LSR to a linear line given by y = 0.094x + 78 and *r* (correlation coefficient) = 0.94.

A


(kDa)
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97.2
66.4
44.3
TLN
29.0
20.1
14.3

B

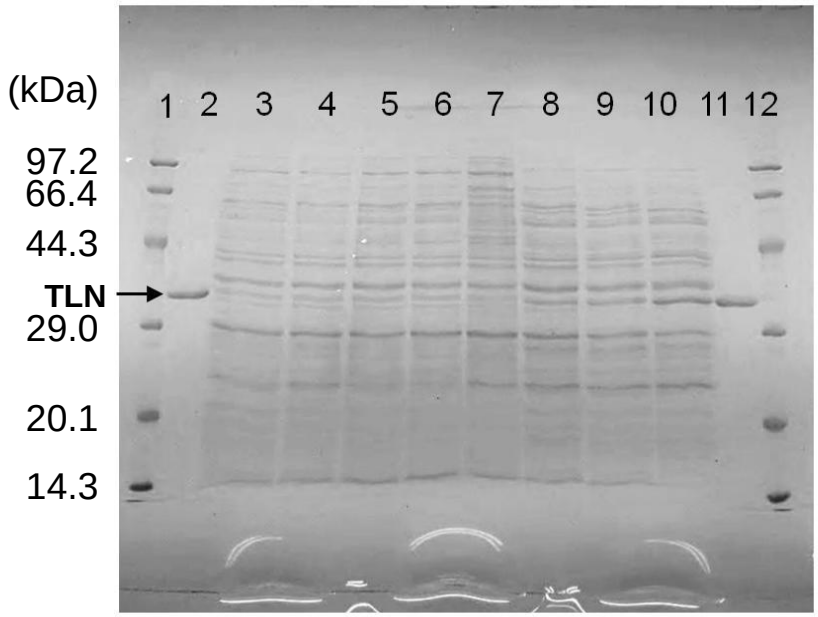
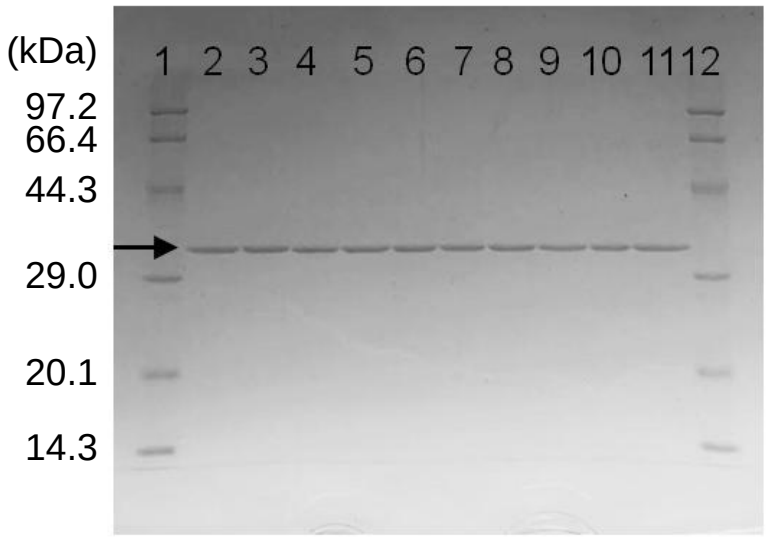

(kDa)
1 2 3 4 5 6 7 8 9 10 11 12
97.2
66.4
44.3
TLN
29.0
20.1
14.3

Fig. 1

A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97.2
66.4
44.3
29.0
20.1
14.3

B

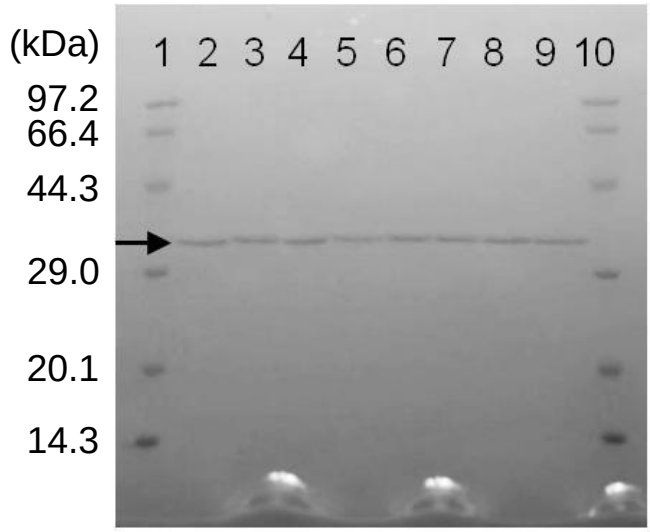
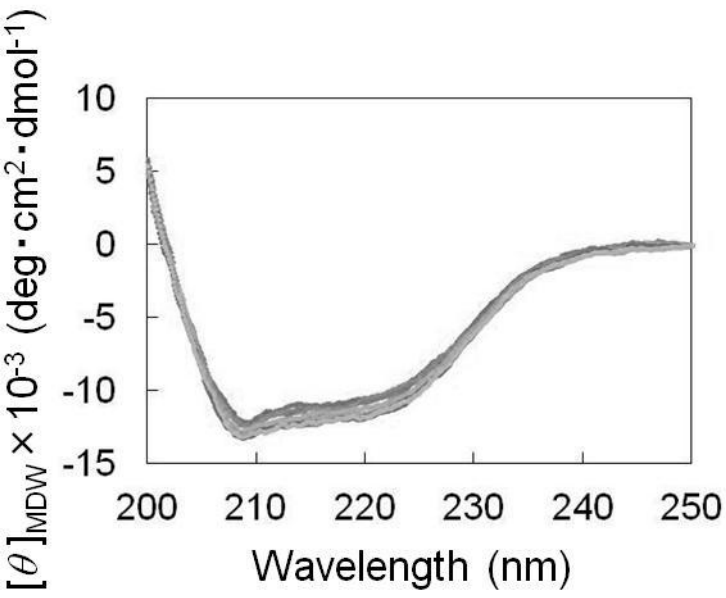

(kDa)
1 2 3 4 5 6 7 8 9 10
97.2
66.4
44.3
29.0
20.1
14.3

Fig. 2

A


10
5
0
-5
-10
-15
200
210
220
230
240
250
Wavelength (nm)
$[\theta]_{MDW} \times 10^{-3}$ (deg·cm²·dmol⁻¹)

B

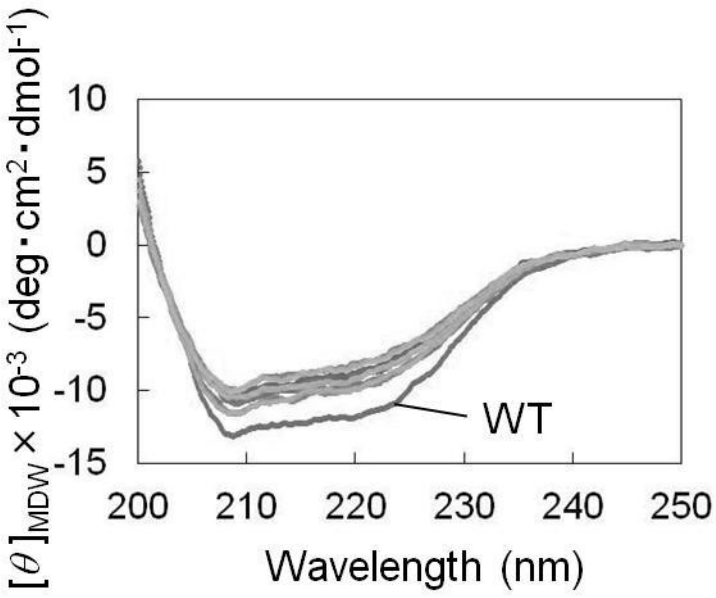

10
5
0
-5
-10
-15
200
210
220
230
240
250
Wavelength (nm)
$[\theta]_{MDW} \times 10^{-3}$ (deg·cm²·dmol⁻¹)
WT

Fig. 3

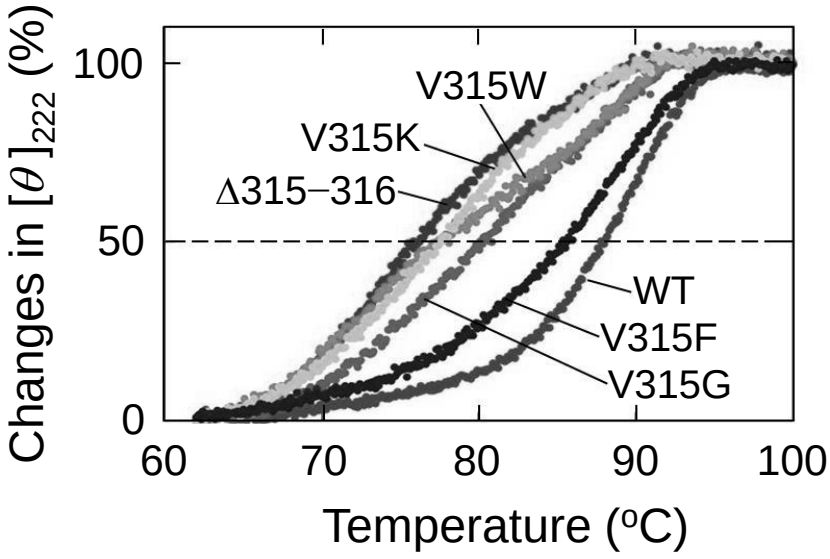

Changes in $[\theta]_{222}$ (%)
100
50
0
60
70
80
90
100
Temperature (°C)
V315W
V315K
Δ315−316
WT
V315F
V315G

Fig. 4

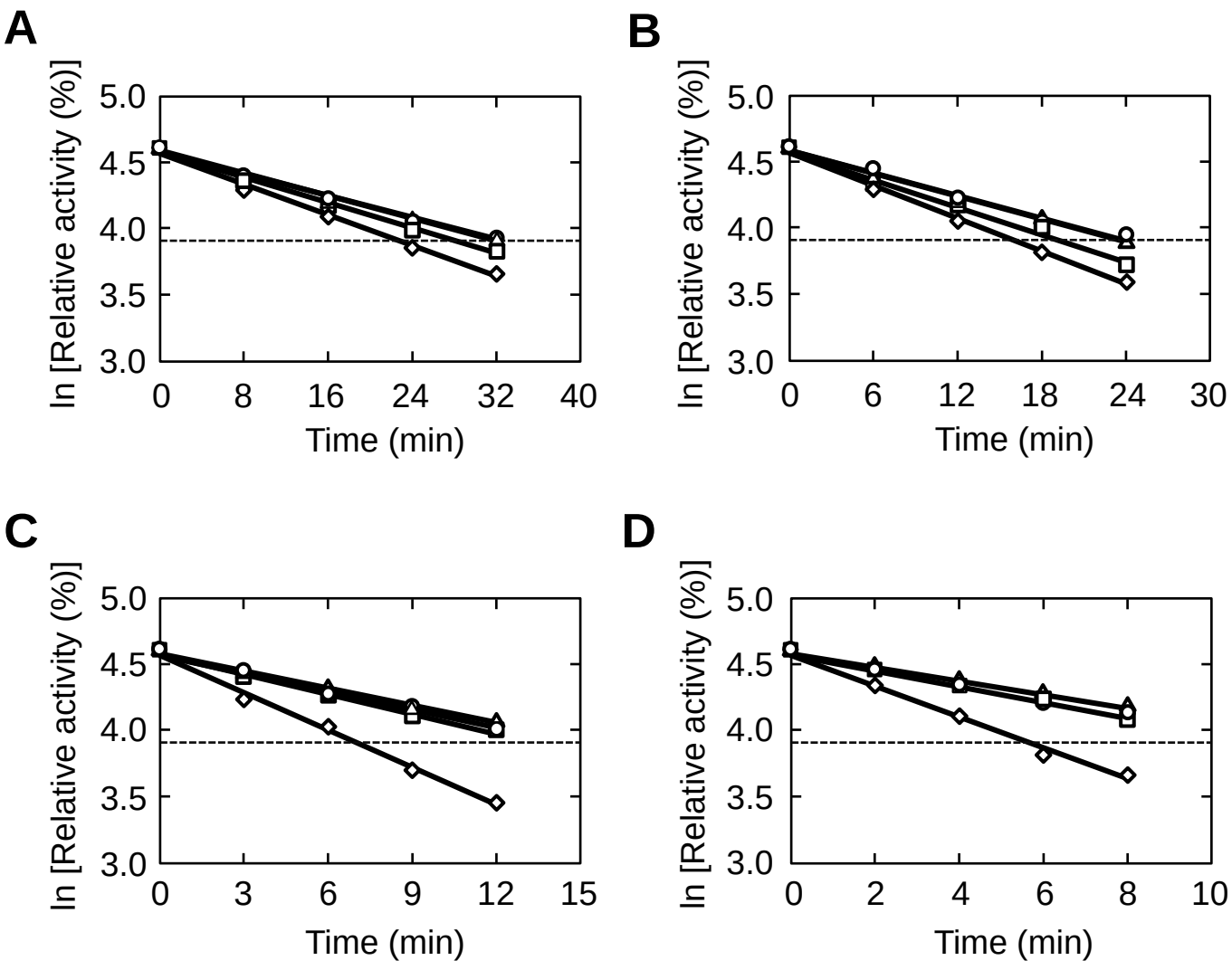
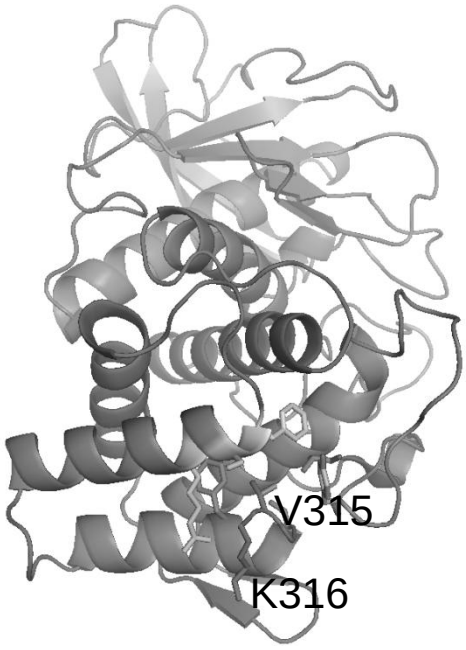

A
B
C
D
ln [Relative activity (%)]
Time (min)
5.0
4.5
4.0
3.5
3.0
0
8
16
24
32
40
6
12
18
30
3
9
15
2
10

Fig. 5

A


V315
K316

B

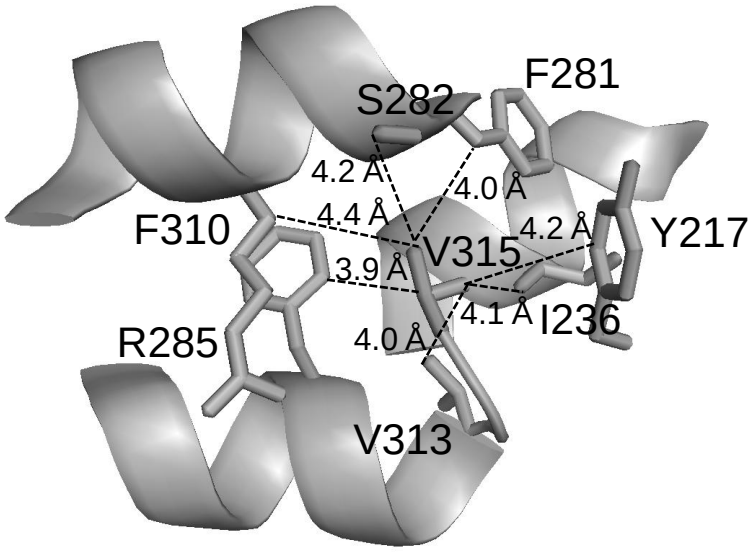

S282
F281
4.2 Å
4.0 Å
4.4 Å
F310
V315
4.2 Å
Y217
3.9 Å
4.1 Å
I236
R285
4.0 Å
V313

Fig. 6

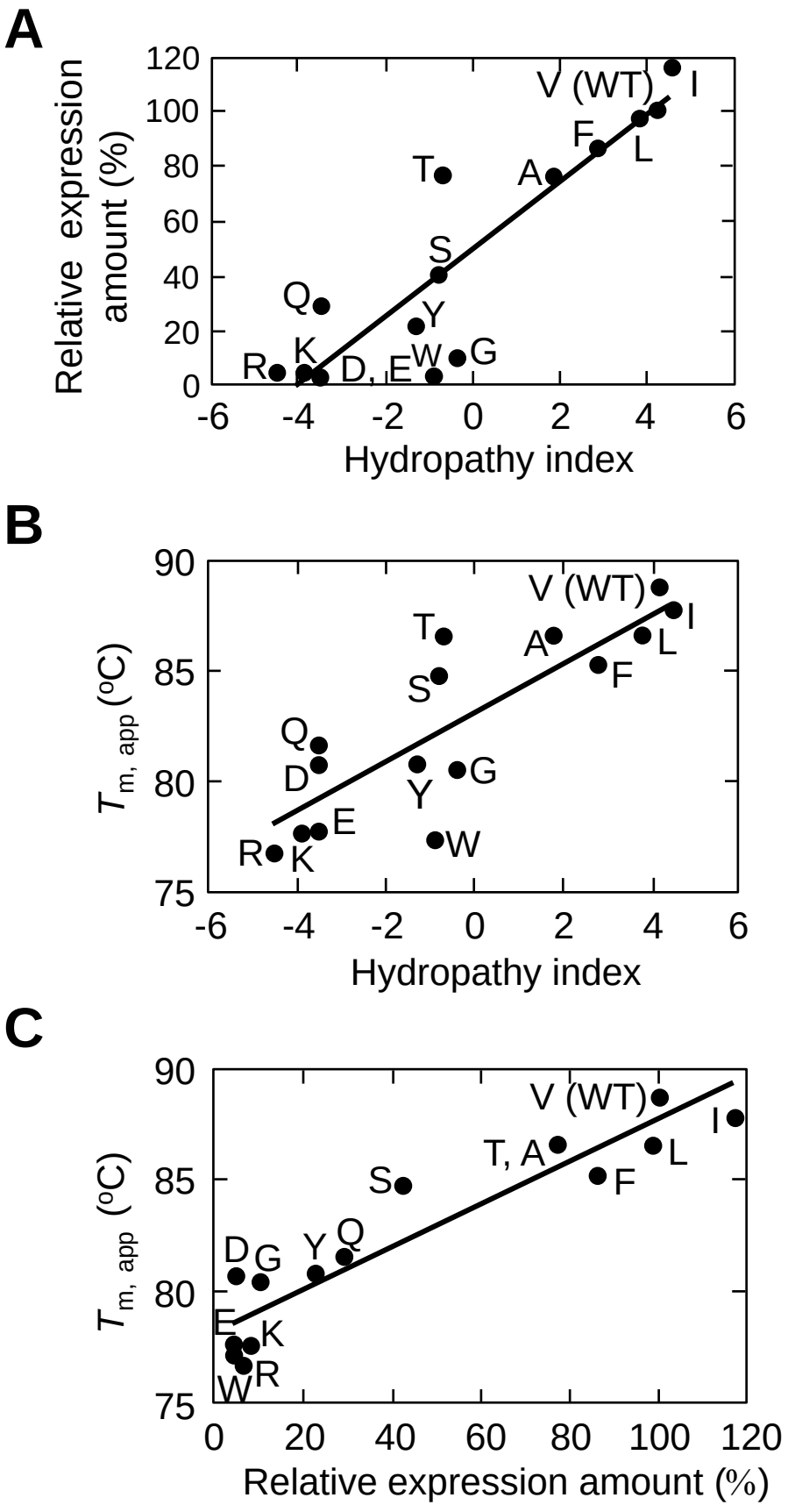

A
Relative expression amount (%)
120
100
80
60
40
20
0
-6
-4
-2
0
2
4
6
Hydropathy index
V (WT)
I
F
L
T
A
S
Q
Y
K
R
D, E
W
G
B
$T_{m,\ app}$ (°C)
90
85
80
75
V (WT)
I
T
A
L
S
F
Q
D
G
Y
E
R
K
W
C
$T_{m,\ app}$ (°C)
90
85
80
75
0
20
40
60
80
100
120
Relative expression amount (%)
V (WT)
I
T, A
L
S
F
D
G
Y
Q
E
K
R
W

Fig. 7

TABLES

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.

Table 2. Casein-hydrolysis activities and expression amounts of WT and thermolysin variants in the culture supernatants of *E. coli* transformed with their expression plasmids, and α-helix contents and $T_{m, app}$ values of the variants.

Thermolysin	[a]Casein-hydrolysis activity in culture supernatant (PU/ml-supernatant)	[b]Casein-hydrolysis activity of purified thermolysin (PU/mg)	[c]Expression amount in culture supernatant (mg/ml-supernatant)	$T_{m, app}$ (^oC)	α-Helix content (%)	Hydropathy index of the residue at position 315
WT	121 ± 1 (1.0)	13 ± 1 (1.0)	9.3 ± 0.7 (1.0)	88.8	36 (1.0)	4.2
Δ316	126 ± 1 (1.0)	13 ± 1 (1.0)	9.7 ± 0.8 (1.0)	87.8 (-1.0)	34 (1.0)	4.2
Δ315–316	ND	11 ± 1 (0.8)	ND	76.0 (-12.8)	25 (0.7)	–
Δ314–316	ND	ND	ND	ND	ND	–
Δ313–316	ND	ND	ND	ND	ND	–
Δ312–316	ND	ND	ND	ND	ND	–
Δ298–316	ND	ND	ND	ND	ND	–
V315G	10 ± 1 (0.1)	11 ± 1 (0.8)	0.9 ± 0.1 (0.1)	80.5 (-8.3)	29 (0.8)	-0.4
V315A	86 ± 1 (0.7)	12 ± 1 (0.9)	7.2 ± 0.6 (0.8)	86.6 (-2.2)	32 (0.9)	1.8
V315L	110 ± 1 (0.9)	12 ± 1 (0.9)	9.2 ± 0.8 (1.0)	86.6 (-2.2)	36 (1.0)	3.8
V315I	120 ± 1 (1.0)	11 ± 1 (0.8)	10.9 ± 1.0 (1.2)	87.8 (-1.0)	31 (0.9)	4.5
V315F	80 ± 1 (0.7)	10 ± 1 (0.8)	8.0 ± 0.8 (0.9)	85.3 (-3.5)	31 (0.9)	2.8
V315Y	25 ± 1 (0.2)	12 ± 1 (0.9)	2.1 ± 0.2 (0.2)	80.8 (-8.0)	34 (1.0)	-1.3
V315W	3 ± 1 (0.02)	8 ± 1 (0.6)	0.4 ± 0.1 (0.04)	77.3 (-11.5)	24 (0.7)	-0.9
V315S	47 ± 1 (0.4)	12 ± 1 (0.9)	3.9 ± 0.3 (0.4)	84.8 (-4.0)	34 (0.9)	-0.8
V315T	93 ± 1 (0.8)	13 ± 1 (1.0)	7.2 ± 0.6 (0.8)	86.6 (-2.2)	36 (1.0)	-0.7
V315Q	27 ± 1 (0.2)	10 ± 1 (0.8)	2.7 ± 0.3 (0.3)	81.6 (-7.2)	27 (0.8)	-3.5
V315D	4 ± 1 (0.03)	10 ± 1 (0.8)	0.4 ± 0.1 (0.04)	80.8 (-8.0)	22 (0.6)	-3.5
V315E	4 ± 1 (0.03)	11 ± 1 (0.8)	0.4 ± 0.1 (0.04)	77.7 (-11.1)	25 (0.7)	-3.5
V315R	5 ± 1 (0.04)	9 ± 1 (0.7)	0.6 ± 0.1 (0.1)	76.7 (-12.1)	28 (0.8)	-4.5
V315K	5 ± 1 (0.04)	7 ± 1 (0.5)	0.7 ± 0.1 (0.1)	77.6 (-11.2)	22 (0.6)	-3.9

ND, not detected (detection limit was 2 PU/ml); Hydrolysis of casein was carried out in 40 mM Tris-HCl buffer (pH 7.5) at 25^{o}C. Numbers in parentheses indicate values relative to WT. The average of triplicate determination with SD value is shown. [a]Culture supernatants (500 ml) collected by the cultivation at 37^{o}C for 48 h were analyzed for casein-hydrolysis activities. [c]Expression amount (mg/ml-supernatant) of thermolysin variants was calculated in dividing the value of the column a by that of the column b.

Table 3. FAGLA-hydrolysis activities of purified thermolysin in the absence and presence of 4 M NaCl.

Thermolysin	$(k_{cat}/K_m) \times 10^{-4}$ (M^{-1} s^{-1})		Degree of activation (B)/(A)
	0 M NaCl (A)	4 M NaCl (B)	
WT	2.3 ± 0.2 (1.0)	39 ± 1 (1.0)	17 (1.0)
Δ316	2.1 ± 0.1 (0.9)	38 ± 3 (1.0)	18 (1.1)
Δ315–316	1.5 ± 0.1 (0.7)	22 ± 1 (0.6)	15 (0.9)
V315G	1.7 ± 0.1 (0.7)	29 ± 1 (0.7)	17 (1.0)
V315A	2.3 ± 0.1 (1.0)	40 ± 3 (1.0)	17 (1.0)
V315L	2.0 ± 0.1 (0.9)	36 ± 1 (0.9)	18 (1.1)
V315I	1.9 ± 0.2 (0.8)	36 ± 1 (0.9)	18 (1.1)
V315F	2.0 ± 0.2 (0.9)	33 ± 1 (0.8)	17 (1.0)
V315Y	2.1 ± 0.1 (0.9)	36 ± 2 (0.9)	17 (1.0)
V315W	1.0 ± 0.1 (0.4)	18 ± 1 (0.5)	18 (1.1)
V315S	2.5 ± 0.1 (1.1)	41 ± 1 (1.1)	16 (0.9)
V315T	2.5 ± 0.1 (1.1)	40 ± 2 (1.0)	16 (0.9)
V315Q	2.1 ± 0.1 (0.9)	36 ± 3 (0.9)	17 (1.0)
V315D	1.5 ± 0.1 (0.7)	22 ± 1 (0.6)	15 (0.9)
V315E	1.5 ± 0.1 (0.7)	26 ± 1 (0.7)	17 (1.0)
V315R	2.1 ± 0.1 (0.9)	37 ± 1 (0.9)	18 (1.1)
V315K	1.8 ± 0.1 (0.8)	31 ± 1 (0.8)	17 (1.0)

FAGLA-hydrolysis activities were measured at 25°C in 40 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ (buffer B*) in the absence and presence of NaCl. The average of triplicate determination with SD value is shown. Numbers in parentheses indicate values relative to WT.