Biosci. Biotechnol. Biochem., **74** (12), 2457–2462, 2010

Effects of Site-Directed Mutagenesis of the Loop Residue of the N-Terminal Domain Gly117 of Thermolysin on Its Catalytic Activity

Evans MENACH, Kiyoshi YASUKAWA, and Kuniyo INOUYE†

Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

Received July 27, 2010; Accepted September 28, 2010; Online Publication, December 7, 2010
[doi:10.1271/bbb.100536]

In the N-terminal domain of thermolysin, two polypeptide strands, Asn112-Ala113-Phe114-Trp115 and Ser118-Gln119-Met120-Val121-Tyr122, are connected by a short loop, Asn116-Gly117, to form an anti-parallel β-sheet. The Asn112-Trp115 strand is located in the active site, while the Ser118-Tyr122 strand and the Asn116-Gly117 loop are located outside the active site. In this study, we explored the catalytic role of Gly117 by site-directed mutagenesis. Five variants, G117A (Gly117 is replaced by Ala), G117D, G117E, G117K, and G117R, were produced by co-expressing in *Escherichia coli* the mature and pro domains as independent polypeptides. The production levels were in the order G117E > wild type > G117K, G117R > G117D. G117A was hardly produced. This result is in contrast to our previous one that all 72 active-site thermolysin variants were produced at the similar levels whether they retained activity or not (M. Kusano *et al. J. Biochem.*, 145, 103–113 (2009)). G117E exhibited lower activity in the hydrolysis of *N*-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide and higher activity in the hydrolysis of *N*-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester than the wild-type thermolysin. G117K and G117R exhibited considerably reduced activities. This suggests that Gly117 plays an important role in the activity and stability of thermolysin, presumably by affecting the geometries of the Asn112-Trp115 and Ser118-Tyr122 strands.

Key words: activity; glycine; metalloproteinase; site-directed mutagenesis; thermolysin

Thermolysin (EC 3.4.24.27) is a thermostable neutral metalloproteinase produced in the culture broth of *Bacillus thermoproteolyticus*.[1–3] It requires one zinc ion for enzyme activity and four calcium ions for structural stability,[4–6] and catalyzes specifically the hydrolysis of peptide bonds containing hydrophobic amino acid residues.[7,8] Thermolysin consists of 316 amino acid residues, and the amino acid sequence has been determined.[9] It consists of a β-rich N-terminal domain and an α-helical C-terminal domain.[10–12] The active site of thermolysin is composed of one zinc ion and five polypeptide regions: an N-terminal strand (Asn112-Trp115), α-helix 1 (Val139-Thr149), C-terminal loop 1 (Asp150-Gly162), α-helix 2 (Ala163-Val176), and C-terminal loop 2 (Gln225-Ser234).[13] The α-helix 1 is located at the bottom of the active site cleft, containing the zinc-coordinating motif ^{142}HEXXH and connecting the N- and C-terminal domains. The activity and stability of thermolysin markedly increase at high concentrations (1–5 M) of neutral salts,[8,14–18] although the mechanisms have not been well elucidated.

Thermolysin is widely used in peptide bond formation through the reverse reaction of hydrolysis, in particular *N*-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester (ZDFM), a precursor of the artificial sweetener aspartame, from *N*-carbobenzoxy-L-aspartic acid (ZD) and L-phenylalanine methyl ester (FM).[14,19] Improvement of its activity and stability and modification of its pH-activity profile are thus still important goals. Site-directed mutagenesis studies of thermolysin have been done extensively, especially on its active-site residues.[20–28] Of the active-site residue variants, L144S, D150E, and I168A have higher activities than wild-type thermolysin (WT) in the hydrolysis of *N*-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide (FAGLA), a widely used substrate for thermolysin,[14,29] and ZDFM. L155A has higher stability than WT.[23,24] N112D,[25] F114H,[13] and Q225A[26] have modified pH-activity profiles.

In the N-terminal domains of thermolysin, two parallel polypeptide strands, Asn112-Ala113-Phe114-Trp115 and Ser118-Gln119-Met120-Val121-Tyr122, are connected by a short loop, Asn116-Gly117, to form an anti-parallel β-sheet (Fig. 1). The Asn112-Trp115 strand is located in the active site, while the Ser118-Tyr122 strand and the Asn116-Gly117 loop are located outside the active site. The objective of this study was to explore the catalytic role of Gly117 by site-directed mutagenesis. Thermolysin variants were expressed, purified, and characterized for their activities and thermal stabilities in the hydrolysis of FAGLA and ZDFM.

Materials and Methods

Materials. Bovine milk casein of Hammerstein grade (lot WKL1761) was purchased from Wako Pure Chemical (Osaka, Japan). FAGLA (lot 111K1764) was purchased from Sigma (St. Louis, MO). The concentrations of FAGLA and ZDFM were determined spectrophotometrically using the molar absorption coefficients, $\varepsilon_{345} = 766\ \text{M}^{-1}\,\text{cm}^{-1}$ and $\varepsilon_{257} = 387\ \text{M}^{-1}\,\text{cm}^{-1}$, respectively.[14,18]

Bacterial strains, plasmids, and transformation. E. coli K12 JM109, *recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, Δ(*lac-proAB*), *F′*(*traD36*, *proAB*$^+$ *lac*^{Iq}, *lacZ*Δ*M15*), was used. pTMP1 is an

† To whom correspondence should be addressed. Tel: +81-75-753-6266; E-mail: inouye@kais.kyoto-u.ac.jp
Abbreviations: FAGLA, *N*-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide; ZDFM, *N*-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester

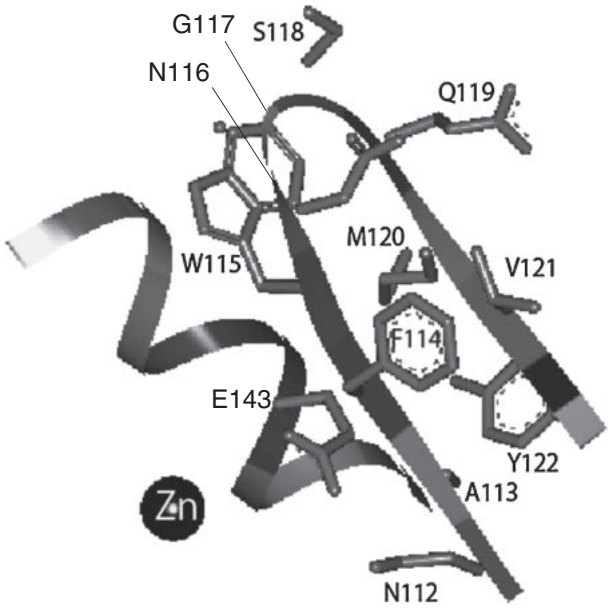

Fig. 1. Close-Up View of the Active Site of Thermolysin.
The structure is based on Protein Data Bank no. 8TLN. The main chain is represented by a ribbon model. Side chains of the residues of the Asn112-Ala113-Phe114-Trp115 strand, the Asn116-Gly117 loop, the Ser118-Gln119-Met120-Val121-Tyr122 strand, and the catalytically important residue Glu143 are represented by ball and stick.

expression plasmid that co-expresses the mature sequence of thermolysin which contains the pelB leader sequence at its N-terminus and the pre-prosequence of thermolysin, as described previously.[30] Site-directed mutagenesis was carried out using a Quikchange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). The nucleotide sequences of the mutated thermolysin genes were verified with a Shimadzu DNA sequencer DSQ-2000 (Shimadzu, Kyoto, Japan). JM109 cells were transformed with the resulting plasmids and cultured in L broth. Ampicillin was used at a concentration of 50 μg/ml.

Production of thermolysin variants. Thermolysin variants were expressed in the supernatant of *E. coli* cultures and purified to homogeneity by sequential column chromatography of the supernatant, as described previously.[30–32] Briefly, for seed culture, 5 ml of L broth in a 20-ml test tube was inoculated with glycerol stock of transformed JM109 cells and grown with shaking at 37 °C for 12 h. The culture (5 ml) was diluted 100 times with 500 ml of L broth in a 1-liter flask and incubated at 37 °C for 48 h, with 0.1% w/v anti-foam A (Sigma) and vigorous aeration with an air pump. The supernatant was applied to a column packed with Toyopearl Phenyl-650M gel (Tosoh, Tokyo). Active fractions were pooled and then applied to a column of glycyl D-phenylalanine coupled to CNB-activated Sepharose 4B resin (Amersham-Pharmacia Biotech, Uppsala, Sweden). Prior to kinetic measurement, the preparations were desalted using pre-packed PD-10 gel filtration columns (Amersham Biosciences, Uppsala, Sweden).

SDS–PAGE. SDS–PAGE was performed in a 12.5% polyacrylamide gel under reducing conditions by the method of Laemmli.[33] A constant current of 40 mA was applied for 40 min. Supernatants were reduced by treatment with 2.5% 2-mercaptoethanol at 100 °C for 10 min. Proteins were stained with Coomassie Brilliant Blue R-250. A molecular-mass marker kit consisting of rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66.3 kDa), rabbit muscle aldolase (42.4 kDa), bovine erythrocyte carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa), and hen egg white lysozyme (14.4 kDa) was from Daiichi Pure Chemicals (Tokyo).

Two-dimensional gel electrophoresis. Isoelectric focusing (IEF) was done with agarGel (Atto, Tokyo), containing carrier ampholytes (pH 3–10) and discRun (Atto). A constant voltage of 300 V was applied for 210 min. Purified wild-type thermolysin or G117E (50 μl of 2 μg/ml in 60 mM Tris–HCl pH 8.9, 5 M urea, 1 M thiourea, 1% v/v 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), 1% v/v Triton X-100, and 1% w/v DTT) was applied to agarGel. After IEF, the gels were expelled from the tubes and equilibrated in 2.5% w/v trichloroacetic acid for 3 min, followed by immersion in water. They were then placed on top of a 12.5% polyacrylamide gel, and SDS–PAGE was done as described above.

Hydrolysis of casein. Casein-hydrolyzing activity was measured by methods described previously.[3,26] The thermolysin solution (0.5 ml) was added to 1.5 ml of a solution containing 1.33% w/v casein and 40 mM Tris–HCl pH 7.5, and incubated at 25 °C for 30 min. The reaction was stopped by the addition of 2 ml of a solution containing 0.11 M trichloroacetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid. After 1-h of incubation at 25 °C, the reaction mixture was filtered through Whatman no. 2 filter paper (70 mm in diameter), and the absorbance (A_{275}) at 275 nm was measured. One proteolytic unit (PU) of activity was 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 (A_{275} of 1 μg of tyrosine)/min.

Spectrophotometric analysis of the thermolysin-catalyzed hydrolysis of FAGLA. Thermolysin-catalyzed hydrolysis of FAGLA was measured by the decrease in absorbance (A_{345}) at 345 nm.[8,14] The amount of FAGLA hydrolyzed was evaluated using the molar absorption difference due to hydrolysis, $\Delta\varepsilon_{345} = -310\ \mathrm{M^{-1}\,cm^{-1}}$, at 25 °C.[8,14,28] The reaction was carried out in 40 mM acetate-NaOH buffer at pH 4.0–5.5, 40 mM MES-NaOH buffer at pH 5.5–7.0, 40 mM HEPES-NaOH buffer at pH 7.0–8.5, and TAPS-NaOH buffer at pH 8.0–9.0, each of which contained 10 mM $CaCl_2$, at 25 °C. Hydrolysis was carried out under pseudo first-order conditions, where the substrate concentration is much lower than the Michaelis constant (K_m) (>30 mM)[14] because of the slight solubility (<6 mM) of FAGLA.[8,14,28] Under the conditions, the kinetic parameters, K_m and the molecular activity (k_{cat}), cannot be determined separately, and enzyme activity was evaluated by the specificity constant (k_{cat}/K_m). The intrinsic k_{cat}/K_m, ($(k_{cat}/K_m)_o$), and the proton dissociation constants (K_{e1} and K_{e2}) for the bell-shaped pH-dependence of the activity (k_{cat}/K_m) were calculated from eq. (1) by a non-linear least-squares regression method with Kaleida Graph Version 3.5 (Synergy Software, Essex, VT):

$$(k_{cat}/K_m)_{obs} = (k_{cat}/K_m)_o/\{1 + ([H]/K_{e1}) + (K_{e2}/[H])\} \qquad (1)$$

In this equation, $(k_{cat}/K_m)_{obs}$ and [H] are the k_{cat}/K_m value observed and the proton concentration respectively at a specified pH, and K_{e1} and K_{e2} correspond to the pK_as in the acidic and alkaline sides of the pH-dependence curve of $(k_{cat}/K_m)_{obs}$.

Spectrophotometric analysis of the thermolysin-catalyzed hydrolysis of ZDFM. Thermolysin-catalyzed hydrolysis of ZDFM was measured by following the decrease in absorbance (A_{224}) at 224 nm.[14] The amount of ZDFM hydrolyzed was measured using the molar absorption difference due to hydrolysis, $\Delta\varepsilon_{224} = -493\ \mathrm{M^{-1}\,cm^{-1}}$, at 25 °C.[14] The reaction was carried out with thermolysin in 40 mM Tris–HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ at 25 °C. The kinetic parameters, k_{cat} and K_m, were determined with Kaleida Graph Version 3.5, based on the Michaelis-Menten equation using the non-linear least-squares method.[34]

Thermal inactivation of thermolysin. Thermolysin (0.5–8 μM) in 40 mM HEPES-NaOH buffer pH 7.5 containing 10 mM $CaCl_2$ was incubated at 80 °C for 0–24 min. Then it was incubated at 25 °C for 1 min. The remaining activity of thermolysin toward FAGLA hydrolysis was determined as described above. Under the assumption that thermal inactivation of thermolysin is irreversible and consists of only one step,[27,28,35] the first-order rate constant, k_{obs}, of thermal inactivation was evaluated by plotting the logarithm of the residual activity (k_{cat}/K_m) against the duration of thermal treatment.

Results

Production of Gly117 variants to homogeneity

Gly117 was changed into one of the negatively charged amino acids (Asp and Glu), one of the positively charged ones (Lys and Arg), or an uncharged

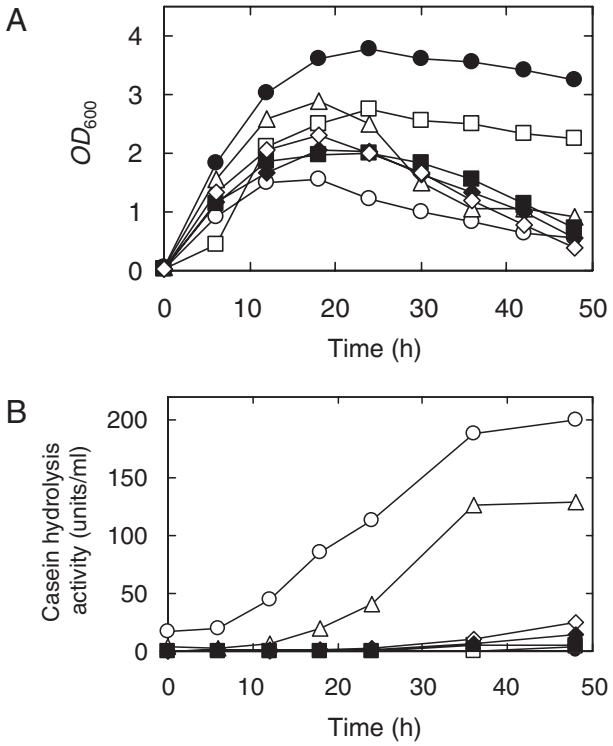

Fig. 2. Fermentation of *E. coli*.

A, Cell densities (OD_{600}) of the culture. B, Casein hydrolysis activity of the supernatants. JM109 cells transformed with pUC19 (solid circle) and expression plasmids for the wild-type thermolysin (open circle), G117A (open square), G117D (solid square), G117E (open triangle), G117K (open diamond), and G117R (solid diamond) are cultured. Zero hour means start of test tube-shake culture.

one (Ala). Wild-type thermolysin (WT) and variants were expressed in *E. coli* and purified from the supernatants.

Figure 2 shows a time course for the cultures of the transformants. In the transformants with the expression plasmids for WT and G117E, casein hydrolysis activities appeared in the supernatants and increased progressively even after OD_{600} reached maximum. In the transformants with the expression plasmid for G117D, G117K, and G117R, low casein hydrolysis activity appeared in the supernatants and increased slightly with time. In the transformants with the expression plasmid for G117A, casein hydrolysis activities did not appear in the supernatants.

On SDS–PAGE of the culture supernatants of the *E. coli* cells transformed with the expression plasmids for WT and the Gly117 variants under reducing conditions, the 34-kDa protein band was clearly detected for WT, G117E, and G117K, but was hardly detected for G117A, G117D, or G117R (data not shown). Figure 3 shows SDS–PAGE of the purified WT and the Gly117 variants, except for G117A. The thermolysin thus obtained yielded a single band with a molecular mass of 34 kDa. From 380–850 ml of the culture supernatants, 1.1 mg of WT, 0.04 mg of G117D, 1.8 mg of G117E, 0.10 mg of G117K, and 0.10 mg of G117R were recovered. Purified G117A was not obtained. Isoelectric focusing showed that the pI values of WT and G117E were 5.3 and 4.8 respectively (data not shown), indicating that the pI value of thermolysin shifted by pH 0.5 unit due to the mutation of Gly117→Glu.

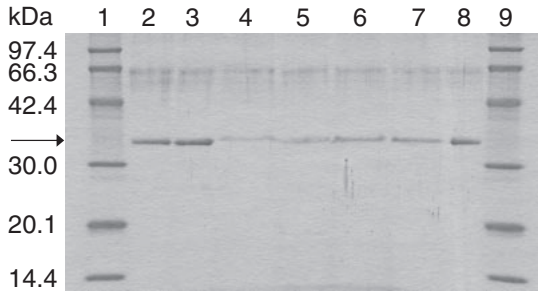

Fig. 3. Coomassie Brilliant Blue-Stained 12.5% SDS–PAGE Gels.

Marker proteins (lanes 1 and 9), native thermolysin purified from *B. thermoproteolyticus* (lanes 2 and 8), WT (lane 3), G117D (lane 4), G117E (lane 5), G117K (lane 6), and G117R (lane 7). The arrow indicates the band corresponding to mature thermolysin.

Characterization of Gly117 variants

The specific activities of WT, G117D, G117E, G117K, and G117R in the hydrolysis of casein were 11,000, 2,000, 12,000, 3,200, and 2,900 units/mg respectively, indicating that the specific activity of G117E was almost the same as that of WT, while those of G117D, G117K, and G117R were 20–30% of that of WT. We did not attempt further characterization of G117D due to a shortage of purified enzyme.

Figure 4A shows the pH-dependence of the k_{cat}/K_m of the thermolysin-catalyzed hydrolysis of FAGLA at 25 °C. All the plots showed bell-shaped curves, with an optimal pH around 7. The $(k_{cat}/K_m)_o$, pK_{e1}, and pK_{e2} values are summarized in Table 1. The $(k_{cat}/K_m)_o$ values for G117E, G117K, and G117R were 80, 40, and 40% respectively of that of WT. The pK_{e1} and pK_{e2} values of the variants were similar to that of WT, except for the pK_{e2} value for G117R (8.6 ± 0.1), which was higher by 0.4 ± 0.3 units than that for WT (8.2 ± 0.2).

Figure 4B shows the dependence of the initial reaction rate (v_o) of the thermolysin-catalyzed hydrolysis of ZDFM at pH 7.5 at 25 °C on the substrate concentration. All the plots showed saturated profiles. The k_{cat} and K_m values are summarized in Table 2. The k_{cat}/K_m values of G117E, G117K, and G117R were 130, 80, and 50% respectively of that of WT. The high k_{cat}/K_m value of G117E was ascribed to its high k_{cat} value.

We have reported that thermolysin activity increases with increasing concentration of neutral salts such as NaCl, and that the degree of NaCl-induced activation, which is defined as the ratio of the k_{cat}/K_m value at 4 M NaCl to that at 0 M NaCl, is in the range of 13–15.[15] Table 3 shows the k_{cat}/K_m values at 0 and 4 M NaCl in the hydrolysis of FAGLA and the degrees of NaCl-induced activation. The degrees of activation of G117E, G117K, and G117R were 90, 30, and 50% respectively of that of WT.

Figure 4C shows the time-dependence of thermal inactivation of the thermolysin variants at 80 °C. Inactivation followed pseudo first-order kinetics. The observed first-order rate constants, k_{obs}, for the thermal inactivation reaction are summarized in Table 4. The order for k_{obs} was G117K > G117R > G117E > WT. Thus thermal inactivation of thermolysin at 80 °C was enhanced in the variants in the order G117K > G117R > G117E.

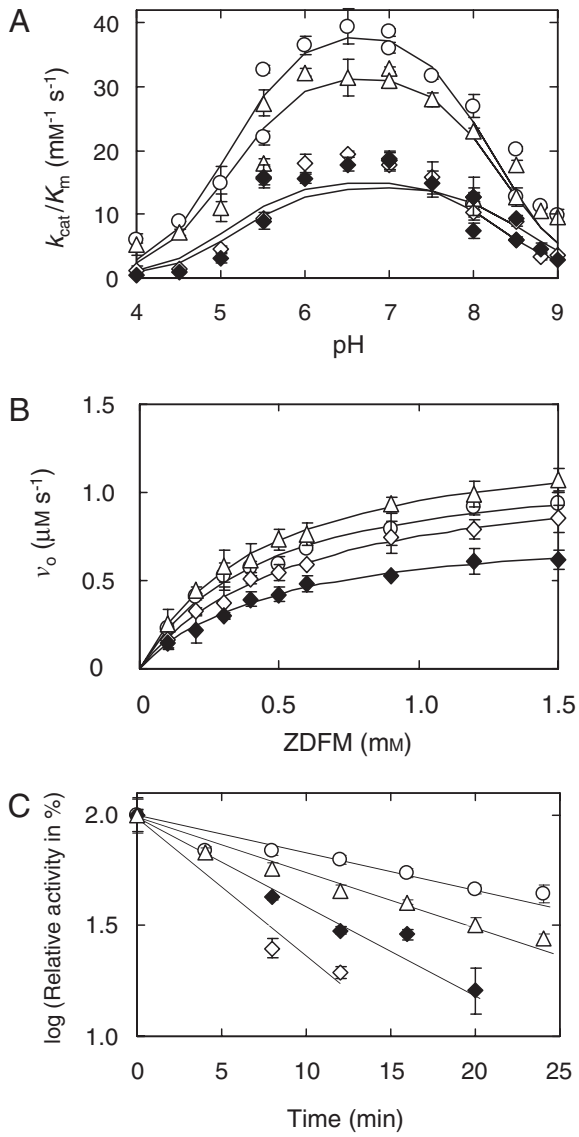

Fig. 4. Characterization of Thermolysin Variants.

A, Effect of pH on the initial reaction rate (v_o) in the thermolysin-catalyzed hydrolysis of FAGLA. The reaction was carried out with initial concentrations of enzyme and FAGLA of 100 nM and 400 μM respectively at 25 °C. B, Dependence on the substrate concentration of v_o in the thermolysin-catalyzed hydrolysis of ZDFM. The reaction was carried out with an initial enzyme concentration $[E]_o$ of 100 nM at 25 °C. C, Thermal inactivation of thermolysins. Thermolysin (1 μM) in 40 mM HEPES-NaOH buffer (pH 7.5) containing 10 mM $CaCl_2$ was incubated at 80 °C for 0–24 min. The FAGLA-hydrolytic reaction was carried out with initial concentrations of enzyme and FAGLA of 100 nM and 400 μM respectively at 25 °C. The remaining activity (k_{cat}/K_m) was expressed as the relative value to that of the intact enzyme (WT, 26 mM^{-1} s^{-1}; G117E, 25 mM^{-1} s^{-1}; G117K, 16 mM^{-1} s^{-1}; and G117R, 15 mM^{-1} s^{-1}) and plotted against the incubation time. Symbols for enzymes: wild-type thermolysin, open circle; G117E, open triangle; G117K, open diamond; and G117R, solid diamond. Error bars indicate SD values for triplicate measurements.

Discussion

Altered substrate specificity of G117E

In thermolysin, the S1 subsite is constituted by Phe114, and the S1′ subsite by Phe130, Leu133, Val139, Ile188, Gly189, Val192, and Leu202.[10,11,21,25] Crystallographic analysis of the complex of thermolysin and an analog of the tetrahedral intermediate carbobenzoxy-Gly^P-L-Leu-L-Leu ("Gly^P" indicates the tetrahedral phosphorus of a phosphonamidate moiety corresponding to the trigonal carbon of the peptide linkage) revealed the postulated hydrogen bonds between thermolysin and the substrate, ND2 of Asn112 and O of the P2′ residue, OD1 of Asn112 and N of the P2′ residue, O of Ala113 and N of P1′ Leu, and N of Trp115 and O of P2 residue.[36] Based on these findings, we speculate as follows: (i) Thermolysin variants with altered substrate specificity are obtained if the geometry of the S1 and S1′ subsites can be changed without much affecting the backbone structure of the Asn112-Trp115 strand, and (ii) the geometry of the Asn112-Trp115 strand can be changed by mutation of Gly117, because the Asn116-Gly117 loop connects the Asn112-Trp115 strand and the Ser118-Tyr122 strand, and Gly is the most flexible

Table 1. pK_e Values and Intrinsic k_{cat}/K_m ($(k_{cat}/K_m)_o$) of Wild-Type Thermolysin and Its Variants in the Hydrolysis of FAGLA at 25 °C

Thermolysin	pK_{e1}	pK_{e2}	$(k_{cat}/K_m)_o$ (mM^{-1} s^{-1})
WT	5.1 ± 0.2 (0.0)	8.2 ± 0.2 (0.0)	40 ± 3 (1.0)
G117E	5.1 ± 0.2 (0.0)	8.3 ± 0.1 (+0.1)	33 ± 2 (0.8)
G117K	5.1 ± 0.1 (0.0)	8.3 ± 0.1 (+0.1)	16 ± 3 (0.4)
G117R	5.2 ± 0.1 (+0.1)	8.6 ± 0.1 (+0.4)	15 ± 2 (0.4)

Average of triplicate determinations with SD values is shown. Numbers in parentheses indicate ΔpK_e as compared to wild-type thermolysin (WT) and the $(k_{cat}/K_m)_o$ relative to WT.

Table 2. Kinetic Parameters of the Wild-Type Thermolysin and Its Variants in the Hydrolysis of ZDFM at 25 °C

Thermolysin	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
WT	0.42 ± 0.04 (1.0)	7.9 ± 0.3 (1.0)	18.8 ± 1.9 (1.0)
G117E	0.44 ± 0.03 (1.0)	10.5 ± 0.3 (1.3)	23.9 ± 2.4 (1.3)
G117K	0.57 ± 0.05 (1.4)	8.4 ± 0.3 (1.1)	14.7 ± 1.4 (0.8)
G117R	0.50 ± 0.04 (1.2)	4.8 ± 0.2 (0.6)	9.6 ± 0.9 (0.5)

Average of triplicate determinations with SD values is shown. Numbers in parentheses indicate values relative to wild-type thermolysin (WT).

Table 3. Degree of Salt-Induced Activation of Wild-Type Thermolysin and Its Variants in the Hydrolysis of FAGLA at 25 °C

Thermolysin	k_{cat}/K_m (mM^{-1} s^{-1})		(B/A)
	0 M NaCl (A)	4 M NaCl (B)	
WT	40 ± 3 (1.0)	600 ± 20 (1.0)	15 (1.0)
G117E	33 ± 2 (0.8)	476 ± 16 (0.8)	14 (0.9)
G117K	16 ± 3 (0.4)	60 ± 2 (0.1)	4 (0.3)
G117R	15 ± 2 (0.4)	105 ± 1 (0.2)	7 (0.5)

The reaction was carried out in 40 mM HEPES-NaOH buffer at pH 7.5, containing 10 mM $CaCl_2$, at 25 °C. Average of triplicate determinations with the SD value is shown. Numbers in parentheses indicate values relative to wild-type thermolysin (WT).

Table 4. Thermal Stability of Wild-Type Thermolysin and Its Variants

Thermolysin	$k^a_{obs} \times 10^4$ (s^{-1})
WT	6.3 ± 0.4 (1.0)
G117E	9.6 ± 0.5 (1.5)
G117K	25 ± 2 (4.0)
G117R	14 ± 2 (2.2)

[a] k_{obs} is the observed first-order rate constant for thermal inactivation at 80 °C. The average of triplicate determinations with the SD value is shown. Numbers in parentheses indicate values relative to wild-type thermolysin (WT).

amino acid residue.

The specific activities of G117E and WT in the hydrolysis of casein were almost the same, as described in "Results." However, the k_{cat}/K_m value in the hydrolysis of FAGLA of G117E was slightly lower than that of WT (Fig. 4A, Table 1), while the k_{cat}/K_m value in the hydrolysis of ZDFM of G117E was slightly higher than that of WT (Fig. 4B, Table 2). The enhanced k_{cat}/K_m value of G117E in the hydrolysis of ZDFM is ascribable to an increase in k_{cat}. FAGLA and ZDFM are substrates routinely used in the characterization of thermolysin[3,14] and neutral proteases from *Bacillus stearothermophilus*.[37–39] FAGLA is a poorly soluble neutral dipeptide, while ZDFM is a negatively charged dipeptide. We have reported several thermolysin variants, L144S, D150E, and I168A, with higher FAGLA- and ZDFM-hydrolyzing activities than WT.[13,27,28] On the other hand, G117E is the first thermolysin variant with lower FAGLA-hydrolyzing and higher ZDFM-hydrolyzing activities. Our strategy appeared to be effective in altering the substrate specificity of thermolysin.

Decreased production levels of G117A and G117D

G117A was not produced at detectable levels in the supernatant of the *E. coli* transformants (Figs. 2 and 3). For their expression, we used the *E. coli* expression system, which does not require autocatalytic cleavage, in which the mature domain of thermolysin, containing an NH_2-terminal pectate lyase B leader sequence and the pre-prodomain of thermolysin, were co-expressed constitutively in *E. coli* as independent polypeptides under the original promoter sequences in the *npr* gene, which encodes thermolysin.[30] Indeed, all 72 active-site thermolysin variants were produced at similar levels by this system whether they retained activity or not.[30] Considering that Ala is more hydrophobic than Gly, the introduced Ala117 might be oriented into a polar environment or outside the protein core, rendering the protein unstable.

G117D was produced at detectable levels in the supernatant of the *E. coli* transformants, but was scarcely purified (Figs. 2 and 3). Asp and Glu have similar characteristics: the pK_as of their side chains in the proteins are 4.4–4.6, their hydrophobicity scores are −3.5, and their volumes are 125 and 155 Å^3 respectively.[40] There is no explanation at this stage why the mutation Gly117→Glu is favorable, while Gly117→Asp is not.

Decreased activities and stabilities of G117K and G117R

In the hydrolysis of FAGLA and ZDFM, the k_{cat}/K_m values of G117K and G117R were in a range of 40–60% of those of WT (Tables 1 and 2), their degrees of the salt-induced activation were 30–50% of that of WT (Table 3), and their k_{obs} values for thermal inactivation at 80 °C were 220–400% of that of WT (Table 4). We speculate that the positive charge introduced at position 117 affects the geometry of the Asn112-Trp115 strand and decreases the activity and stability of thermolysin. In regard to this, according to the crystal structure of thermolysin (Protein Data Bank no. 8TLN), the distance OD2 of Asp150 and Cα of Gly117 is only 4.2 Å. Asp150 is located at C-terminal loop 1 (Asp150-Gly162) in the active site. We have found that D150E showed higher activity, while D150H and D150W showed higher stability.[13] We assume that introduced Lys or Arg at position 117 interacts with Asp150, rendering the protein less active and stable.

Thermolysin is surely a representative zinc metalloproteinase with high activity and stability. However, if its substrate specificity can be altered as we hope, it can provide many benefits to industry. Recent crystallographic analyses of aminopeptidase N have revealed that aminopeptidase N and thermolysin are strikingly similar in the structure of the C-terminal, but not the N-terminal, domain.[41–43] There is a possibility that aminopeptidase activity can be generated in thermolysin by introducing negative charges that can recognize the positive charges of the N-terminal amino nitrogen of the substrate. In conclusion, Gly117 plays an important role in the activity and stability of thermolysin, presumably by affecting the geometry of the Asn112-Trp115 and Ser118-Tyr122 strands. Site-directed mutagenesis experiments on Asn116 are currently underway.

Acknowledgments

This study was supported in part by Grants-in-Aid for Scientific Research (nos. 17380065 and 20380061, to K.I.) from the Japan Society for the Promotion of Science.

References

1) Endo S, *J. Ferment. Technol.* (in Japanese), **40**, 346–353 (1962).
2) van den Burg B and Eijsink VG, "Handbook of Proteolytic Enzymes" 2nd ed. Vol. 1, eds. Barrett JA, Rawlings ND, and Woessner JF, Elsevier, Amsterdam, pp. 374–387 (2004).
3) Inouye K, "Handbook of Food Enzymology," eds. Whitaker JR, Voragen AGJ, and Wong DWS, Marcel Dekker, New York, pp. 1019–1028 (2003).
4) Latt SA, Holmquist B, and Vallee BL, *Biochem. Biophys. Res. Commun.*, **37**, 333–339 (1969).
5) Feder J, Garrett LR, and Wildi BS, *Biochemistry*, **10**, 4552–4556 (1971).
6) Tajima M, Urabe I, Yutani K, and Okada H, *Eur. J. Biochem.*, **64**, 243–247 (1976).
7) Morihara K and Tsuzuki H, *Eur. J. Biochem.*, **15**, 374–380 (1970).
8) Inouye K, Lee S-B, and Tonomura B, *Biochem. J.*, **315**, 133–138 (1996).
9) Titani K, Hermodson MA, Ericsson LH, Walsh KA, and Neurath H, *Nature*, **238**, 35–37 (1972).
10) Holmes MA and Matthews BW, *J. Mol. Biol.*, **160**, 623–639 (1982).
11) Hangauer DG, Monzingo AF, and Matthews BW, *Biochemistry*, **23**, 5730–5741 (1984).
12) Mock WL and Aksamawati M, *Biochem. J.*, **302**, 57–68 (1994).
13) Kusano M, Yasukawa K, and Inouye K, *J. Biochem.*, **145**, 103–113 (2009).
14) Inouye K, *J. Biochem.*, **112**, 335–340 (1992).
15) Inouye K, Lee S-B, Nambu K, and Tonomura B, *J. Biochem.*, **122**, 358–364 (1997).
16) Inouye K, Kuzuya K, and Tonomura B, *Biochim. Biophys. Acta*, **1388**, 209–214 (1998).
17) Inouye K, Kuzuya K, and Tonomura B, *J. Biochem.*, **123**, 847–852 (1998).
18) Oneda H, Muta Y, and Inouye K, *Biosci. Biotechnol. Biochem.*, **68**, 1811–1813 (2004).
19) Oyama K, Kihara K, and Nonaka Y, *J. Chem. Soc., Perkin II*, 356–360 (1981).

20) Toma S, Campagnoli S, de Gregoriis E, Gianna R, Margarit I, Zamai M, and Grandi G, *Protein Eng.*, **2**, 359–364 (1989).
21) Inouye K, Mazda N, and Kubo M, *Biosci. Biotechnol. Biochem.*, **62**, 798–800 (1998).
22) Hanzawa S and Kidokoro S, "Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation," eds. Flickinger MC and Drew SW, John Wiley & Sons, New York, pp. 2527–2535 (1999).
23) Matsumiya Y, Nishikawa K, Aoshima H, Inouye K, and Kubo M, *J. Biochem.*, **135**, 547–553 (2004).
24) Matsumiya Y, Nishikawa K, Inouye K, and Kubo M, *Lett. Appl. Microbiol.*, **40**, 329–334 (2005).
25) Kusano M, Yasukawa K, Hashida Y, and Inouye K, *J. Biochem.*, **139**, 1017–1023 (2006).
26) Tatsumi C, Hashida Y, Yasukawa K, and Inouye K, *J. Biochem.*, **141**, 835–842 (2007).
27) Yasukawa K and Inouye K, *Biochim. Biophys. Acta*, **1774**, 1281–1288 (2007).
28) Kusano M, Yasukawa K, and Inouye K, *J. Biotechnol.*, **147**, 7–16 (2010).
29) Feder J, *Biochem. Biophys. Res. Commun.*, **32**, 326–332 (1968).
30) Yasukawa K, Kusano M, and Inouye K, *Protein Eng. Des. Sel.*, **20**, 375–383 (2007).
31) Inouye K, Minoda M, Takita T, Sakurama H, Hashida Y, Kusano M, and Yasukawa K, *Protein Expr. Purif.*, **46**, 248–255 (2006).
32) Yasukawa K, Kusano M, Nakamura K, and Inouye K, *Protein Expr. Purif.*, **46**, 332–336 (2006).
33) Laemmli UK, *Nature*, **227**, 680–685 (1970).
34) Sakoda M and Hiromi K, *J. Biochem.*, **80**, 547–555 (1976).
35) Takita T, Aono T, Sakurama H, Itoh T, Wada T, Minoda M, Yasukawa K, and Inouye K, *Biochim. Biophys. Acta*, **1784**, 481–488 (2008).
36) Tronrud DE, Holden HM, and Matthews BW, *Science*, **235**, 571–574 (1987).
37) Eijsink VG, van den Burg B, Vriend G, Berendsen HJ, and Venema G, *Biochem. Int.*, **24**, 517–525 (1991).
38) Mansfeld J and Ulbrich-Hofmann R, *Biotechnol. Bioeng.*, **97**, 672–679 (2006).
39) Durrschmidt P, Mansfeld J, and Ulbrich-Hofmann R, *Biophys. Chem.*, **147**, 66–73 (2010).
40) Taylor WR, *J. Theor. Biol.*, **119**, 205–218 (1986).
41) Kyrieleis OJ, Goettig P, Kiefersauer R, Huber R, and Brandstetter H, *J. Mol. Biol.*, **349**, 787–800 (2005).
42) Addlagatta A, Gay L, and Matthews BW, *Proc. Natl. Acad. Sci. USA*, **103**, 13339–13344 (2006).
43) Ito K, Nakajima Y, Onohara Y, Takeo M, Nakashima K, Matsubara F, Ito T, and Yoshimoto T, *J. Biol. Chem.*, **281**, 33664–33676 (2006).