Effects of site-directed mutagenesis in the N-terminal domain of thermolysin on its stabilization

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Yuichi Kawasaki, Kiyoshi Yasukawa and Kuniyo Inouye*

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

*Kuniyo Inouye. Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan. Tel: +81-75-753-6266, Fax: +81-75-753-6265, email: inouye@kais.kyoto-u.ac.jp

The thermolysin variant G8C/N60C/S65P in which the triple mutation in the N-terminal domain, Gly8→Cys/Asn60→Cys/Ser65→Pro, is undertaken increases stability [Yasukawa, K. and Inouye, K. (2007) Improving the activity and stability of thermolysin by site-directed mutagenesis. *Biochim. Biophys. Acta* 1774, 1281–1288] and its mechanism is examined in this study. The apparent denaturing temperatures based on ellipticity at 222 nm of the wild-type thermolysin (WT), G8C/N60C, S65P and G8C/N60C/S65P were 85, >95, 88 and >95°C, respectively. The first-order rate constants, k_{obs} , of the thermal inactivation of WT and variants at 10 mM CaCl₂ increased with increasing thermal treatment temperatures (70–95°C), and those at 80°C decreased with increasing CaCl₂ concentrations (1–100 mM). The k_{obs} values were in the order of WT>S65P>G8C/N60C=G8C/N60C/S65P at all temperatures and CaCl₂ concentrations. These results indicate that the mutational combination, Gly8→Cys/Asn60→Cys and Ser65→Pro, increases stability only as high as Gly8→Cys/Asn60→Cys does. Assuming that irreversible inactivation of thermolysin occurs only in the absence of calcium ions, the dissociation constants, K_d , to the calcium ions of WT, G8C/N60C, S65P and G8C/N60C/S65P were 47, 8.9, 17 and 7.2 mM, respectively, suggesting that Gly8→Cys/Asn60→Cys and Ser65→Pro stabilize thermolysin by improving its affinity to calcium ions, most probably the one at the Ca²⁺-binding site III in the N-terminal domain.

Keywords: calcium ion/metalloproteinase/site-directed mutagenesis/stability/thermolysin.

Abbreviations: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; FAGLA, *N*-[3-(2-furyl)-acryloyl]-glycyl-L-leucine amide; TNB, thiobis(2-nitrobenzoic acid).

amino acid residues with one zinc ion essential for enzyme activity and four calcium ions required for structural stability (3–6). Based on the structural data (7, 8), it consists of a β -rich N-terminal domain and an α -helical C-terminal domain. The Ca²⁺-binding sites I, II and IV are located in the C-terminal domain, and the Ca²⁺-binding site III is located in the N-terminal domain (7, 8) (Fig. 1A). When thermolysin is incubated with ethylenediamine-*N,N,N',N'*-tetraacetic acid (EDTA), all calcium ions are removed from the thermolysin molecule and autolysis occurs (9–11). Thermolysin catalyses specifically the hydrolysis of peptide bonds containing hydrophobic amino acid residues (12, 13). It is widely used for the peptide bond formation through reverse reaction of hydrolysis, in particular, *N*-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester (ZDFM), the precursor of an artificial sweetener aspartame, from *N*-carbobenzoxy-L-aspartic acid (ZD) and L-phenylalanine methyl ester (FM) (2, 14). Improvement of its activity and stability and modification of its pH-activity profile are thus still important goals (15). We have reported that high concentrations (1–5 M) of neutral salts increase the activity and stability of thermolysin (13, 14, 16, 17).

Site-directed mutagenesis experiments of thermolysin (18–26) and thermolysin-like protease (TLP-ste) [EC 3.4.24.4] (27, 28) have generated a number of variant enzymes with improved activity and/or stability. TLP-ste is a neutral metalloproteinase from *Bacillus stearothermophilus*. It consists of 319 amino acid residues and differs from thermolysin at 44 out of 319 residues. (In this study, the amino acid sequence of thermolysin is adopted for numbering that of TLP-ste.) In TLP-ste, a single mutation Ser65→Pro was first reported to increase stability (27). (In this study, the mutation of a residue, e.g. Ser65 to Pro is designated as Ser65→Pro, and the variant enzyme bearing Ser65→Pro is designated as S65P.) After that, a double mutation Gly8→Cys/Asn60→Cys, designed to introduce a disulphide bridge between the amino acid residues 8 and 60, was reported to increase stability (28). In thermolysin, we reported that the mutational combination of Gly8→Cys/Asn60→Cys and Ser65→Pro increases stability without affecting activity (20). These results suggest that the N-terminal domain is important for stability in thermolysin and TLP-ste. However, little is known about the mechanism of stabilization by these mutations.

In this study, we examined the effects of the mutations Gly8→Cys/Asn60→Cys and Ser65→Pro on the thermal denaturation and inactivation of thermolysin. Our results suggest that Gly8→Cys/Asn60→Cys and Ser65→Pro stabilize thermolysin by improving its affinity to calcium ions.

Thermolysin [EC 3.4.24.27] is a thermostable neutral metalloproteinase produced in the culture broth of *Bacillus thermoproteolyticus* (1, 2). It consists of 316

Fig. 1 Structure of thermolysin. (A) Whole structure of the WT. The overall protein structure (ribbon model), the mutated residues (ball and stick model) and zinc and calcium ions (sphere) are shown. (B) Close-up view of the Ca²⁺-binding site III of WT. The Ca3, all residues, water molecules are shown in ball and stick model. The distances between the Ca3 and each of the six oxygen atoms are in the range of 2.3–2.5 Å. (C) Close-up view of the N-terminal domain (Ile1–Ala70) of WT, G8C/N60C and S65P. The overall protein structure (wire model) and the Ca3, Gly8 or Cys8, Asn60 or Cys60, Ser65 or Pro65, Asp57, Asp59, Asn60 and Gln61 (ball and stick model) are shown. The structure of WT is based on PDB code 8TLN (8). The structures of G8C/N60C and S65P are based on protein modelling with a Swiss model using the structure of WT as a template.

Materials and Methods

Materials

Casein (lot PEH5596) was purchased from Wako Pure Chemical (Osaka, Japan). *N*-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide (FAGLA) (lot 111K1764) was from Sigma (St. Louis, MO, USA). The concentration of FAGLA was determined spectrophotometrically using the molar absorption coefficient, $\epsilon_{345} = 766 \text{ M}^{-1} \text{ cm}^{-1}$ (14, 29). 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (lot MOG7777) and mineral oil (lot M9R1743) were from Nacalai Tesque (Kyoto, Japan).

Bacterial strains, plasmids and transformation

Escherichia coli K12 JM109 [*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, Δ (*lac-proAB*), *F*(*traD36*, *proAB*⁺ *lacI*^q, *lacZAM15*)] was used. pTMP1 is an expression plasmid that co-expresses the mature sequence of thermolysin containing 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 QuikchangeTM site-directed mutagenesis kit

(Stratagene, La Jolla, CA, USA). The nucleotide sequences of mutated thermolysin genes were verified with a Shimadzu DNA sequencer DSQ-2000 (Shimadzu, Kyoto, Japan). JM109 cells were transformed with each of the resulting plasmids and cultured in L broth. The concentration of ampicillin in L broth was 50 µg/ml.

Production of thermolysin

Production of wild-type thermolysin (WT) and its variants was performed as described previously (30). Briefly, for seed culture, 5 ml of L broth in a 20-ml test tube was inoculated with the glycerol stock of the transformed JM109 cells and grown with shaking at 37°C for 12 h. The culture (5 ml) was diluted to 100 times with 500 ml of L broth in a 1-L flask and grown with shaking at 37°C for 48 h. The enzymes were purified to homogeneity by sequential column chromatography procedures of the supernatant of *E. coli* cultures with hydrophobic interaction chromatography, followed by Gly-D-Phe affinity chromatography. Prior to kinetic measurements, the enzyme preparations were desalted using pre-packed PD-10 gel filtration columns (GE Healthcare, Uppsala, Sweden).

SDS-PAGE

SDS-PAGE was performed in a 12.5% polyacrylamide gel under reducing conditions by the method of Laemmli (31). A constant current of 40 mA was applied for 40 min. The enzyme preparations 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* (97.2 kDa), bovine serum albumin (66.4 kDa), hen egg-white ovalbumin (44.3 kDa), bovine carbonic anhydrase (29.0 kDa), soybean trypsin inhibitor (20.1 kDa) and hen egg-white lysozyme (14.3 kDa) was from Takara Bio (Otsu, Japan).

Quantification of disulphide bridges

Thiol quantification was done by the method of Ellman (32). Briefly, 50 µl of 2.5 M dithiothreitol (DTT) or water and 10 µl of 10 mM phosphoramidon were added to 2,440 µl of 11.2 µM thermolysin in 40 mM HEPES–NaOH, 10 mM CaCl₂, pH 7.5. The mixture was incubated at 37°C for 1 h. Then, it was incubated at 100°C for 120 min. To remove excess DTT, the mixture was applied to a pre-packed PD-10 gel filtration column and the elute was collected. Twenty µl of 10 mM DTNB in ethanol was added to 1,980 µl of the elute. The resulted mixture (2,000 µl), which contained 8 µM thermolysin was incubated at 25°C for 1 h. The number of thiol groups modified with DTNB was spectrophotometrically determined from A_{412} with ϵ_{412} of 13,600 M⁻¹cm⁻¹ (32). The number of disulphide bridges was calculated by the difference in number of DTNB-modified thiol groups between the thermolysins incubated at 37°C for 1 h in the presence and the absence of DTT.

Hydrolysis of casein

Casein-hydrolyzing activity was determined according to the methods as described previously (2, 20). Briefly, the thermolysin solution (0.5 ml) was added to 1.5 ml of a solution containing 1.33% (w/v) casein in 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 30-min incubation at 25°C, the reaction mixture was filtered through Whatman No. 2 filter paper (70 mm in diameter), 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 (A_{275} of 1 µg of tyrosine) min⁻¹.

Hydrolysis of FAGLA

Thermolysin-catalysed hydrolysis of FAGLA was carried out in 40 mM HEPES–NaOH, 10 mM CaCl₂, pH 7.5 at 25°C and measured following the decrease in A_{345} (13, 14). The amount of FAGLA hydrolysed was evaluated using the molar absorption difference due to hydrolysis, $\Delta\epsilon_{345} = -310$ M⁻¹cm⁻¹, at 25°C (13, 14, 29). 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) (14) because of the sparing solubility (<6 mM) of FAGLA (13, 14, 29). Under the conditions, the enzyme activity was evaluated by the specificity constant (k_{cat}/K_m).

Circular dichroism measurement

A Jasco J-820 (Tokyo, Japan) spectropolarimeter equipped with a Peltier system of cell temperature control was used. The spectrometer conditions were: spectral range 200–250 nm; 100 mdeg sensitivity; 0.1 nm resolutions; 4 s response time; 20 nm min⁻¹ scan rate and 5 accumulations. The control baseline was obtained with solvent and all the components without thermolysin. The circular dichroism (CD) spectra were recorded at 25°C using 2-mm cell. The concentration of thermolysin was 1.0 µM in 40 mM Tris–HCl, 10 mM CaCl₂, pH 7.5. CD spectra were processed with a Jasco software and finally expressed in mean-residue molar ellipticity units, [θ] (deg cm² dmol⁻¹).

Thermal denaturation of thermolysin

Thermolysin (2.5 µM) in 40 mM HEPES–NaOH, 1 mM CaCl₂, pH 7.5 was incubated at 25°C for 5 min. After the incubation, the solution (400 µl) was transferred to a 2-mm cell and mineral oil (50 µl) was added to avoid evaporation. Thermal denaturation was

examined by monitoring the CD value at 222 nm, θ_{222} , from 70°C to 100°C at 0.5°C min⁻¹. The fraction unfolded (F_u) was determined after normalizing θ_{222} of native and denatured thermolysins between 0 and 1, according to Equation (1).

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

where A_O is the observed θ_{222} of thermolysin at various temperatures and A_N and A_D are θ_{222} of native and denatured enzymes, respectively. The temperature at which F_u is 0.5 is defined as an apparent denaturing temperature.

Thermal inactivation of thermolysin

Thermolysin (200 nM) in 40 mM HEPES–NaOH, 1–100 mM CaCl₂, pH 7.5 was incubated at a set temperature (70–95°C) for specified time (2–120 min). Then, it was incubated at 25°C for 6 min. The relative activity for FAGLA hydrolysis was determined as described above. Assuming that the thermal inactivation of thermolysin is irreversible and consists of only one step, the first-order rate constant, k_{obs} , of the thermal inactivation was evaluated by plotting logarithm of the relative activity (k_{cat}/K_m) against the duration time of thermal treatment. The activation energy, E_a , for the thermal inactivation was determined from an Arrhenius plot according to Equation (2) and the standard Gibbs energy change of activation, $\Delta G^\ddagger$, for the thermal inactivation was determined according to Equation (3) (33)

$$\ln(k_{obs}) = -(E_a/R)(1/T) + \text{Const} \quad (2)$$

$$\Delta G^\ddagger = -RT[\ln(k_{obs}) - \ln(RT/Nh)] \quad (3)$$

where R , T , N and h are the gas constant (=8.314 J K⁻¹ mol⁻¹), absolute temperature in degrees Kelvin, Avogadro number (=6.022 × 10²³ mol⁻¹) and Plank constant (=6.626 × 10⁻³⁴ J s), respectively. The standard enthalpy change of activation, $\Delta H^\ddagger$, for the thermal inactivation was estimated according to Equation (4). Using the estimated $\Delta G^\ddagger$ and $\Delta H^\ddagger$ values at a certain temperature (80°C), the standard entropy change of activation, $\Delta S^\ddagger$, for the thermal inactivation was estimated according to Equation (5).

$$\Delta H^\ddagger = E_a - RT \quad (4)$$

$$\Delta S^\ddagger = (\Delta H^\ddagger - \Delta G^\ddagger)/T \quad (5)$$

Results

Production of thermolysin variants

We constructed three thermolysin variants, G8C/N60C, S65P, G8C/N60C/S65P. Starting with 500 ml of *E. coli* cultures, 0.8–3.0 mg purified enzymes were recovered. On SDS-PAGE under reducing conditions, they yielded a single band with a molecular mass of 34 kDa (Fig. 2A). On CD spectroscopy at 25°C, they exhibited negative ellipticities at around 203–238 nm with the peaks around 208 and 222 nm (Fig. 2B). These results suggest that all variants did not suffer from any drastic structural changes by the mutation.

Casein and FAGLA hydrolysis activities of thermolysin variants

Table I shows hydrolysis activities of thermolysin variants for casein and FAGLA at pH 7.5 at 25°C. The relative activities of the variants to that of the WT were 80–100% for casein and 90–100% for FAGLA. These results indicate that the activities of variants were hardly changed by the mutations.

Quantification of disulphide bridges of thermolysin variants

The disulphide bond formation in G8C/N60C was predicted in protein modelling with a Swiss model (Fig. 1C). When one disulphide bridge is reduced, two thiol groups are produced. To see whether a

Fig. 2 Production of thermolysin variants. (A) SDS–PAGE. Coomassie Brilliant Blue-stained 12.5% SDS–polyacrylamide gel is shown. Purified preparations (300 ng) were applied to each lane. Lane 1, molecular-mass marker; lane 2, WT; lane 3, G8C/N60C; lane 4, G8C/N60C/S65P; lane 5, S65P. The arrow indicates thermolysin. (B) CD spectroscopy at 25°C of WT (thin solid line), G8C/N60C (thick solid line), S65P (dotted line) and G8C/N60C/S65P (dashed line).

Table I. Hydrolysis activities of thermolysin variants for casein and FAGLA at 25°C.

Thermolysin	Casein	FAGLA
	Specific activity (units/mg)	$k_{\text{cat}}/K_m \times 10^{-4}$ ($\text{M}^{-1} \text{s}^{-1}$)
WT	12,000 ± 300 (1.0)	2.2 ± 0.1 (1.0)
G8C/N60C	12,300 ± 100 (1.0)	2.0 ± 0.1 (0.9)
S65P	10,000 ± 100 (0.8)	2.1 ± 0.1 (1.0)
G8C/N60C/S65P	11,000 ± 200 (0.9)	2.1 ± 0.1 (1.0)

Casein hydrolysis reaction was carried out in 40 mM Tris–HCl, pH 7.5 at 25°C. FAGLA hydrolysis reaction was carried out in 40 mM HEPES–NaOH, 10 mM CaCl_2 , pH 7.5 at 25°C. Numbers in parentheses indicate values relative to WT. The average of triplicate determination with standard deviation value is shown.

disulphide bridge is introduced in thermolysin by the mutation Gly8→Cys/Asn60→Cys, the thiol groups of DTT-treated or untreated WT, G8C/N60C and G8C/N60C/S65P (8 μM) were quantified (Table II). The difference in A_{412} of DTNB-modified, and DTT-treated WT and DTNB-modified and DTT-untreated WT was almost zero. The difference in A_{412} of DTNB-modified, and DTT-treated G8C/N60C and DTNB-modified and DTT-untreated G8C/N60C was 0.21, corresponding to 16 μM thiol group. The same result was obtained for G8C/N60C/S65P. This suggests that as predicted in the protein modelling (Fig. 1C), all G8C/N60C and G8C/N60C/S65P molecules have one intra-molecular disulphide bridge.

Thermal denaturation of thermolysin variants

We examined thermal denaturation of WT and variants by monitoring θ_{222} in the range of 70–100°C (Fig. 3). The denaturation curves of WT and S65P showed apparent two-state model. However, the thermal denaturation was not reversible. SDS–PAGE analysis revealed that this was due to autolysis of thermolysin (data not shown). The denaturation curves of G8C/N60C and G8C/N60C/S65P indicated that the entire denaturation of these two variants occurs at higher temperature than 100°C, which is beyond the capacity of temperature control system. The apparent denaturing temperatures of WT,

Table II. Thiol quantification of thermolysin variants.

Thermolysin	A_{412}		
	DTT-treated (A)	DTT-untreated (B)	A–B
WT	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.01
G8C/N60C	0.21 ± 0.01	0.00 ± 0.01	0.21 ± 0.02
G8C/N60C/S65P	0.21 ± 0.01	0.00 ± 0.01	0.21 ± 0.02

The average of triplicate determination with standard deviation value is shown.

G8C/N60C, S65P and G8C/N60C/S65P were 85 ± 1 , >95 , 88 ± 1 , $>95^\circ\text{C}$, respectively, indicating that Gly8→Cys/Asn60→Cys increases stability higher than Ser65→Pro does.

Thermal inactivation of thermolysin variants

We examined the thermal inactivation of thermolysin variants at various temperatures (70–95°C) in the presence of 10 mM CaCl_2 . All inactivations followed pseudo-first-order kinetics (Fig. 4 for 80°C, data not shown for 70, 75, 85, 90 and 95°C). The first-order rate constants, k_{obs} , of the thermal inactivation are summarized in Table III. The k_{obs} values of WT, G8C/N60C, S65P and G8C/N60C/S65P, increased with increasing temperatures from 70°C to 95°C, and were in the order of WT > S65P > G8C/N60C $\approx$ G8C/N60C/S65P at all temperatures examined. This indicates that the mutation Gly8→Cys/Asn60→Cys increases stability higher than the mutation Ser65→Pro does. It also indicates that the combination of the mutations Gly8→Cys/Asn60→Cys and Ser65→Pro increases stability only as high as the mutation Gly8→Cys/Asn60→Cys does. Figure 5 shows Arrhenius plot of k_{obs} . Linear relationship was obtained between the logarithmic value of k_{obs} and $1/T$. Table IV summarizes the thermodynamic parameters for the thermal inactivation of thermolysin at 80°C. This indicates that the mutation Gly8→Cys/Asn60→Cys decreased E_a , $\Delta H^\ddagger$ and $\Delta S^\ddagger$. In other words, the enhanced thermal stability of G8C/N60C and G8C/N60C/S65P was due to the decrease in $\Delta S^\ddagger$.

We examined the thermal inactivation of thermolysin variants at 80°C in the presence of various CaCl_2 concentrations (1–100 mM). The thermolysin variant D150E/I168A with increased activity and decreased stability (23) was also analysed as a comparison. All inactivations followed pseudo-first-order kinetics. The k_{obs} values of WT and variants decreased with increasing CaCl_2 concentrations, and were in the order of D150E/I168A > WT > S65P > G8C/N60C $\approx$ G8C/N60C/S65P at all CaCl_2 concentrations examined (Fig. 6A).

Discussion

Evaluation of enzyme stability

In general, two schemes, Schemes 1 and 2, have been used for evaluating the enzyme stabilities (34).

where N , D and PD represent the native, denatured and partially denatured species, respectively. Scheme 1 is applied to thermodynamic stability, in which the stability of proteins is assessed by ΔG° that represents

Fig. 3 Thermal denaturation of thermolysin variants. θ_{222} of WT and variants were monitored from 70°C to 100°C at 0.5°C min⁻¹. Symbols: WT, open circle; G8C/N60C, open triangle; S65P, open square; G8C/N60C/S65P, open diamond.

the difference in G° between the native and denatured states. Scheme 2 is applied to kinetic stability, in which the stability of proteins is assessed by $\Delta G^{\ddagger}$ that represents the difference in $G^{\ddagger}$ between the native and transition states. In this study, both Gly8→Cys/Asn60→Cys and Ser65→Pro increased ΔG° (Fig. 3) and $\Delta G^{\ddagger}$ (Figs 4–6). Similar results were reported in other enzymes: in *Ochrobactrum* parathion hydrolase, the mutation Gly194→Pro increased T_m from 67.0°C to 70.3°C and the temperature at which the enzyme lost 50% of its activity by 10-min incubation from 62°C to 67°C (35). In *Pseudomonas fluorescens* esterase, the triple mutation Glu81→Asn/Lys86→Arg/Glu87→Asp increased T_m from 73°C to 81°C and the temperature at which the enzyme lost 50% of its activity by 60-min incubation from 63°C to 71°C (36).

Effects of the mutations Gly8→Cys/Asn60→Cys and Ser65→Pro on the binding of thermolysin with calcium ions

Thermolysin has four calcium ions (Ca1–Ca4). Their binding sites are termed as Ca^{2+} -binding sites I–IV, respectively. The Ca^{2+} -binding site III is located in the N-terminal domain and the Ca^{2+} -binding sites I, II and IV are in the C-terminal domain (Fig. 1A). Assuming that (i) one of the four calcium ions is crucial for stability and (ii) irreversible inactivation occurs only from the thermolysin without the calcium ion, the inactivation can be expressed in Scheme 3 (37):

where N is the native thermolysin form containing the calcium ion, and N^* and U^* are the native and inactivated thermolysin forms without the calcium ion, respectively. k_{den} is the rate constant for conversions from N^* to U^* . k_{obs} can be expressed as Equation (6). K_d is the dissociation constant of thermolysin to the calcium ion. K_d can be expressed as Equations (7) and (8) and is obtained from Equations (6) and (7).

$$k_{\text{obs}} = k_{\text{den}} N^* / (N + N^*) \quad (6)$$

Fig. 4 Thermal inactivation of thermolysin variants. FAGLA-hydrolysis activities of WT and variants were measured at 25°C after thermal incubation at 80°C for specified time. The relative activity (k_{cat}/K_m) was expressed as the relative value to that before the incubation at 80°C. The relative activities (A) and their natural logarithms (B) were plotted against incubation time. Symbols: WT, open circle; G8C/N60C, open triangle; S65P, open square; G8C/N60C/S65P, open diamond.

Table III. Observed first-order rate constants (k_{obs}) of thermal inactivation of thermolysin variants.

Thermolysin	$k_{\text{obs}} \times 10^4 \text{ (s}^{-1}\text{)}$					
	70°C	75°C	80°C	85°C	90°C	95°C
WT	1.8 ± 0.3	2.5 ± 0.2	4.4 ± 0.3	12.7 ± 0.6	37.2 ± 6.7	75.1 ± 4.9
G8C/N60C	0.6 ± 0.1	1.5 ± 0.4	1.8 ± 0.1	4.8 ± 0.4	6.1 ± 0.5	9.1 ± 1.3
S65P	1.0 ± 0.1	2.0 ± 0.2	2.8 ± 0.2	7.6 ± 0.1	24.2 ± 2.2	66.3 ± 7.8
G8C/N60C/S65P	0.7 ± 0.1	1.6 ± 0.1	2.4 ± 0.3	3.1 ± 0.5	4.7 ± 0.2	7.9 ± 0.6

The average of triplicate determination with standard deviation value is shown.

Fig. 5 Arrhenius plot of k_{obs} values. The natural logarithms of the k_{obs} values were plotted against the reciprocal of absolute temperature of thermal inactivation. Symbols: WT, open circle; G8C/N60C, open triangle; S65P, open square; G8C/N60C/S65P, open diamond.

Table IV. Thermodynamic parameters for thermal inactivation of thermolysin variants at 80°C.

Thermolysin	E_a (kJ mol ⁻¹)	$\Delta G^\ddagger$ (kJ mol ⁻¹)	$\Delta H^\ddagger$ (kJ mol ⁻¹)	$\Delta S^\ddagger$ (J mol ⁻¹ K ⁻¹)
WT	167 ± 16	110 ± 1	164 ± 13	154 ± 52
G8C/N60C	113 ± 10	112 ± 1	110 ± 7	-7 ± 30
S65P	178 ± 16	111 ± 1	175 ± 13	180 ± 54
G8C/N60C/S65P	93 ± 7	111 ± 1	90 ± 4	-62 ± 18

E_a , $\Delta G^\ddagger$, $\Delta H^\ddagger$ and $\Delta S^\ddagger$ are the activation energy, the Gibbs energy change of activation, the enthalpy change of activation and the entropy change of activation, respectively. The average of triplicate determination with standard deviation value is shown.

$$K_d = [N^*][\text{Ca}^{2+}]/[N] \quad (7)$$

$$1/k_{\text{obs}} = (1/k_{\text{den}}) + [\text{Ca}^{2+}]/(K_d k_{\text{den}}) \quad (8)$$

Equation (8) shows that K_d and k_{den} can be obtained by plotting $1/k_{\text{obs}}$ against $[\text{Ca}^{2+}]$. Figure 6B and Table V show the result of this analysis. The k_{den} values at 80°C of G8C/N60C, S65P and G8C/N60C/S65P were almost the same as that of WT, whereas that of D150E/I168A was slightly different from that of WT. On the other hand, the K_d values at 80°C of G8C/N60C, S65P and G8C/N60C/S65P were much different from that of WT, whereas that of D150E/I168A was almost the same as that of WT. This suggests that Gly8→Cys/Asn60→Cys and Ser65→Pro increase the binding affinity of thermolysin to the calcium ion, but does not influence the stability of the thermolysin without the calcium ion and that

Asp150→Glu/Ile168→Ala does not influence the binding affinity of thermolysin to the calcium ion, but decreases the stability of thermolysin without the calcium ion. We speculate that Gly8→Cys/Asn60→Cys and Ser65→Pro increase thermolysin stability by increasing its affinity to one calcium ion, most probably the Ca3 in the N-terminal domain.

The Ca3 is hexacoordinated with Asp57 (bidentate), Asp59, the main chain carbonyl oxygen atom of Gln61 and two water molecules (Fig. 1B and C). The modelled structures of G8C/N60C and S65P are almost the same as that structure of WT (Fig. 1C), suggesting that neither Gly8→Cys/Asn60→Cys nor Ser65→Pro affects the dynamics rather than the structure of the Ca^{2+} -binding site III.

Role of Ca^{2+} in the stabilization by the mutations Gly8→Cys/Asn60→Cys and Ser65→Pro

Various stabilizing methods have been applied in site-directed mutagenesis of enzymes. These include introduction of a disulphide bridge, formation of static interactions, increases in hydrophobicity, methionine residue replacement, glycine residue replacement, introduction of proline residue and metal binding. Enzymes from thermophiles acquire higher stability than their structurally similar counterparts in mesophiles by a combination of various small effects. According to the summary of characterization of about 700 variants of phage T4 lysozyme (38), most surface residues contribute little to the stability and the number of stabilizing mutations of surface residues is small. It also reveals that if the sites of the two stabilizing mutations are not in contact, the stabilizing effects will be additive. However, these observations are not applicable to thermolysin. Gly8, Asn60 and Ser65 are surface residues in the N-terminal domain. The distance of Cα of Ser65 and Cα of Gly8 is 15.9 Å, and that of Cα of Ser65 and Cα of Asn60 is 14.7 Å (Fig. 1A), indicating that the sites of these two stabilizing mutations are not in contact. In this study, Ser65→Pro stabilizes WT but not G8C/N60C, indicating that the stabilizing effects are not additive. This can be explained that both mutations increase stability with the same mechanism, which we think is the increase in binding affinity to Ca3. A hyperthermostable serine protease Tk-subtilisin from *Thermococcus kodakarensis* contains seven calcium ions. Recent site-directed mutagenesis study of Tk-subtilisin revealed that four calcium ions (Ca2–Ca5) are responsible for folding (39), whereas the other three (Ca1, Ca6 and Ca7) are responsible for stability (40).

Fig. 6 Effect of CaCl_2 concentration on the thermal inactivation of thermolysin variants. Thermolysin (200 nM) in 40 mM HEPES–NaOH, 1–100 mM CaCl_2 , pH 7.5 was incubated at 80°C for a specified time. The subsequent experimental condition corresponds to that of Fig. 4. The first-order rate constant of thermal inactivation of thermolysin (k_{obs}) (A) and $1/k_{\text{obs}}$ (B) were plotted against the CaCl_2 concentration. Symbols: WT, open circle; G8C/N60C, open triangle; S65P, open square; G8C/N60C/S65P, open diamond; D150E/I168A, closed circle.

Table V. Rate constants (k_{den}) for the thermal inactivation and dissociation constants (K_d) to Ca^{2+} of thermolysin variants at 80°C.

Thermolysin	$k_{\text{den}} \times 10^4 \text{ (s}^{-1}\text{)}$	$K_d \text{ (mM)}$
WT	$5.1 \pm 0.6 \text{ (1.0)}$	$47 \pm 10 \text{ (1.0)}$
G8C/N60C	$4.1 \pm 0.8 \text{ (0.8)}$	$8.9 \pm 2.3 \text{ (0.2)}$
S65P	$5.7 \pm 1.5 \text{ (1.1)}$	$17 \pm 6 \text{ (0.4)}$
G8C/N60C/S65P	$4.9 \pm 1.7 \text{ (1.0)}$	$7.2 \pm 2.8 \text{ (0.2)}$
D150E/I168A	$7.3 \pm 0.5 \text{ (1.4)}$	$48 \pm 11 \text{ (1.0)}$

Numbers in parentheses indicate values relative to WT. The average of triplicate determination with standard deviation value is shown.

Such characterization has not been done for the calcium ions of thermolysin.

Previous crystallographic study revealed that when thermolysin was soaked with Co^{2+} , the Ca3 was replaced with Co^{2+} (41). We reported that Co^{2+} not only competitively inhibits thermolysin activity, but also promotes its autolysis and that the former was not suppressed by Ca^{2+} but the latter was suppressed (42). These evidences also suggest that of the four calcium ions, Ca3 is crucial for thermolysin stability.

In conclusion, the mutations Gly8→Cys/Asn60→Cys and Ser65→Pro reduced thermal denaturation and inactivation of thermolysin. It is suggested that Gly8→Cys/Asn60→Cys and Ser65→Pro stabilize thermolysin by improving its affinity to the calcium ion, most probably the Ca3 in the N-terminal domain.

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Conflict of interest

None declared.

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