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Effects of Site-directed Mutagenesis of the Surface Residues Gln128 and Gln225 of Thermolysin on its Catalytic Activity

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Thermolysin is remarkably activated and stabilized by neutral salts with varying degrees depending on salt species, and particular surface residues are thought to be especially important in its activity and stability [Inouye, K. (1992) *J. Biochem.* 112, 335–340; Inouye, K. *et al.* (1998) *Biochim. Biophys. Acta* 1388, 209–214]. In this study, we examined the mutational effects of the surface residues of thermolysin. Gln128 and Gln225 were selected as the residues to be mutated because they are located on the surface loop and close to but not in the active site (23.5 and 15.8 Å far from the active site zinc ion, respectively) and fully solvent accessible. Nine single mutants [Q128K (Gln128 is replaced with Lys), Q128E, Q128A, Q225K, Q225R, Q225E, Q225D, Q225A and Q225V] were constructed by site-directed mutagenesis. Mutational changes in catalytic activity were found only in the mutant thermolysins having a hydrophobic residue at the position 225 (Q225A and Q225V). In the hydrolysis of a neutral substrate *N*-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide (FAGLA), the alkaline pK_a value of Q225A is 8.48 ± 0.04, being higher by 0.42 ± 0.07 units than that of the wild-type thermolysin. The k_{cat}/K_m value of the wild-type enzyme is enhanced 14 times with 4 M NaCl, and those of Q225A and Q225V are enhanced 10 and 19 times, respectively. In the hydrolysis of a negatively charged substrate *N*-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester (ZDFM), unlike FAGLA, the initial velocities of Q225A and Q225V decreased to 30 and 50% of that of the wild-type enzyme, respectively. Their thermal stability is similar to that of the wild-type enzyme. These findings indicate that even a single mutation at the thermolysin surface induces changes in the electrostatic environment in the active site and affects the activity. Thus, site-directed mutagenesis of surface residues of thermolysin, including apparently thermodynamically unfavorable introduction of hydrophobic residues, should be explored to improve its activity and stability.

Key words: expression, metalloproteinase, site-directed mutagenesis, surface residue, thermolysin.

Abbreviations: FAGLA, *N*-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide; *npr*, neutral protease gene; SD, standard deviation; ZDFM, *N*-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester.

Thermolysin [EC 3.4.24.27] is a thermostable neutral metalloproteinase produced in the culture broth of *Bacillus thermoproteolyticus* (*1–4*). It requires one zinc ion for enzyme activity and four calcium ions for structural stability (*5–7*), and catalyses specifically the hydrolysis of peptide bonds containing hydrophobic amino acid residues (*8*, *9*). Thermolysin consists of 316 amino acid residues, and the amino acid sequence was determined (*10*). Thermolysin is widely used for the peptide bond formation through reverse reaction of hydrolysis (*11–13*).

Generally, enzymes from halophilic organisms, as exemplified by malate dehydrogenase from the extreme halophile, *Halobacterium marismortui* (*14*), are halophilic. In addition, particular enzymes from virus, higher plants and non-halophilic organisms have been characterized to be activated by salts. Such enzymes include HIV-1 protease (*15*), actinidain from kiwifruit (*16*) and human matrix metalloproteinase 7 (matrilysin) (*17*). We have reported that thermolysin activity in the hydrolysis and even in the synthesis of peptides are remarkably enhanced by high concentration (1–4 M) neutral salts, and the activity increased typically with increasing the NaCl concentration in an exponential fashion (*12*, *18–21*). It should be of note that the stability is also enhanced by neutral salts (*18*). Thermolysin activity was enhanced 13–15 times with 4 M NaCl in the hydrolysis of a neutral substrate *N*-[3-(2-furyl)acryloyl]-glycyl-L -leucine amide (FAGLA) and 6–7 times with 3.8 M NaCl in the hydrolysis of *N*-carbobenzoxy-L -aspartyl-L -phenylalanine (ZDFM), a negatively charged substrate and a precursor of a synthetic sweetener, aspartame at pH 7.0, at 25°C (*12*, *18–20*). In addition, the solubility of thermolysin was enhanced from 1.0 to 1.2 mg/ml with 0 M NaCl to 10 mg/ml with 2.0–2.5 M NaCl (*21*). Because contents of free water in the solution with such high

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concentrations of NaCl are low, the dielectric constant must be reduced. More importantly, the orders of ions for the efficiency in the activation and the increase in the solubility of thermolysin were $Na^+ > K^+ > Li^+$, which was different from Hofmeister's series corresponding to the degree of hydration of ions: $Li^+ > Na^+ > K^+$ (*9*, *12*, *21*). The degree of the salt-induced activation revealed the bell-shaped pH-dependence with the maximum at pH 7, suggesting an importance of surface charges of thermolysin because the degree of ionization of surface residues is thought to be maximal at pH 7 (*19*). These findings suggest that not only the dielectric constant of the reaction medium but also the interaction between ions and particular surface residues of thermolysin is involved in the salt-induced activation; however, such residues have not been identified. Interestingly, like the activation effects of neutral salts, the inhibitory effects of alcohols on thermolysin activity are shown to be related to the dielectric constant of the reaction medium and the interaction of alcohols with particular residues (*22*, *23*). To explore the mechanism of the salt-induced activation, we recently reported the preliminary X-ray crystallographic analysis of thermolysin in the presence of 4 M NaCl (*24*).

Since the *npr* gene encoding thermolysin was first cloned from *B. thermoproteolyticus* (*25*), site-directed mutagenesis experiments of thermolysin have been extensively performed (*26–31*). Thermolysin-like protease (TLP-ste) [EC 3.4.24.4], a neutral protease from *B. stearothermophilus* that consists of 319 amino acid residues and differs from thermolysin at 44 of 319 residues, has also been used for the same purpose (*32–36*). Glu143 and His231 were demonstrated to play significant catalytic roles (*32*, *33*) as predicted by the structural data (*37–39*). Mutant enzymes with improved activity (*26*, *27*), improved stability (*29*, *30*, *35*, *36*) or modified pH-activity profile (*31*) have been generated by the mutations in the vicinity of the active site or the N-terminal domain of thermolysin. However, little is known about the mutational effects of the surface residues on the enzyme activity and stability.

The objective of this study is to modify the activity and stability of thermolysin by site-directed mutagenesis of the surface residues. For this purpose, we selected Gln128 and Gln225, located 23.5 and 15.8 Å far from the active site zinc ion, respectively, as the residues to be mutated (Fig. 1), based on the following criteria: 1) The mutated residue is located on the surface loop and close to but not in the active site. 2) The mutated residue is not charged but polar and fully solvent accessible. The mutant thermolysins (in this article, a mutant thermolysin in which Gln128 is replaced with Lys is designated Q128K) were expressed, purified and characterized for their activities and thermal stabilities in the hydrolysis of FAGLA and ZDFM.

MATERIALS AND METHODS

Materials—FAGLA (Lot 111K1764) was purchased from Sigma (St Louis, MO, USA). The concentration of FAGLA was determined spectrophotometrically using the molar absorption coefficient, $\varepsilon_{345} = 766\,M^{-1}\,cm^{-1}$ (*12*, *40*).

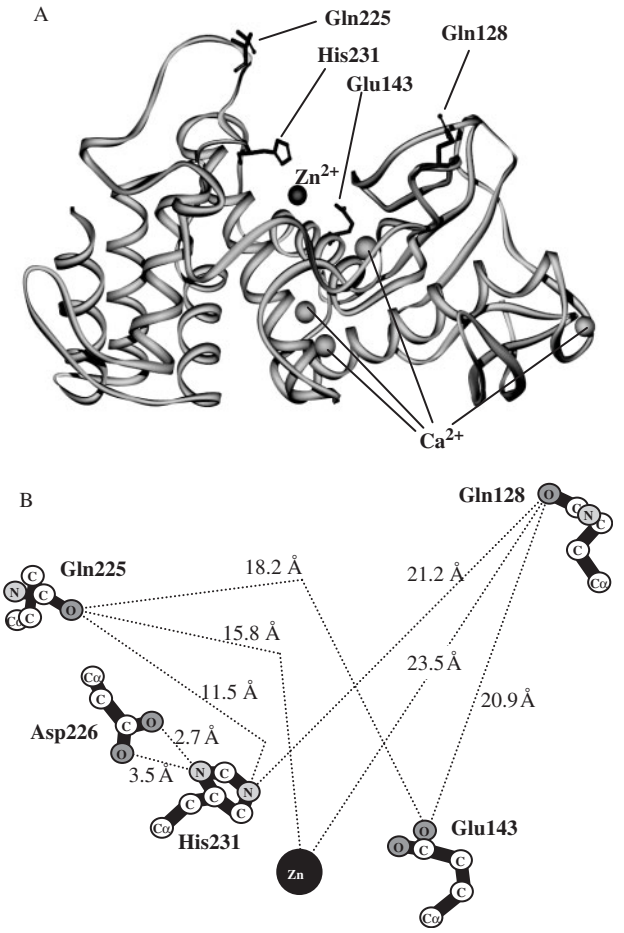

Fig. 1. **Stereochemical relationships between Gln128, Glu143, Gln225, Asp226, His231 and active site Zn^{2+} of thermolysin.** The overall protein structure (A) and the active site (B) of thermolysin (Protein Data Bank number 8TLN) (*51*) are shown.

ZDFM was prepared as described previously (*12*). The concentration of ZDFM was determined using the molar absorption coefficient, $\varepsilon_{257} = 387\,M^{-1}\,cm^{-1}$ (*12*).

Bacterial Strains, Plasmids and Transformation—*E. coli* K12 JM109 [*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1,* Δ(*lac-proAB*), *F'*(*traD36, proAB*$^+$ *lac*^{Iq}, *lacZΔM15*)] was used. pTE1 is an expression plasmid that contains a 2033-bp fragment containing the complete *npr* gene and the promoter region, as described previously (*41*). Site-directed mutagenesis was carried out using a Quikchange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The nucleotide sequences of mutated thermolysin genes were verified by a Shimadzu DNA sequencer DSQ-2000 (Kyoto). JM109 cells were transformed with each of the plasmids and cultured in L broth. Ampicillin was used at the concentration of 50 μg/ml.

Production of Mutant Enzymes—Fermentation and purification of the wild-type thermolysin and the mutant enzymes were performed as described previously (*41*, *42*). Active and mature thermolysin was purified to homogeneity by sequential column-chromatography

procedures of the supernatant of *E. coli* cells with hydrophobic-interaction chromatography followed by affinity chromatography. Prior to kinetic measurements, the preparations were desalted using pre-packed PD-10 gel filtration columns (Amersham Biosciences, Uppsala, Sweden).

Spectrophotometric Analysis of the Thermolysin-catalysed Hydrolysis of FAGLA—Hydrolysis of FAGLA by thermolysin was measured following the decrease in absorbance at 345 nm (*9*, *12*). The amount of FA-dipeptide amides hydrolysed was evaluated using the molar absorption difference due to hydrolysis, $\Delta\varepsilon_{345} = -310\ M^{-1}\ cm^{-1}$, at 25°C (*9*, *12*, *40*). The reaction was carried out in 40 mM acetate–NaOH buffer at pH 4.0–5.4, 40 mM MES buffer at pH 5.2–6.8, 40 mM HEPES buffer at pH 6.8–8.0 and TAPS buffer at pH 7.8–8.8, each of which containing 10 mM $CaCl_2$. The reaction with FAGLA was carried out under pseudo-first-order conditions, where the substrate concentration is lower than the Michaelis constant K_m (>30 mM) (*12*), because of the sparing solubility (<6 mM) of FAGLA (*9*, *12*, *40*). Under the conditions, the kinetic parameters, K_m and the molecular activity k_{cat}, cannot be determined separately, and the enzyme activity was evaluated by the specificity constant, k_{cat}/K_m. The kinetic parameters, the intrinsic k_{cat}/K_m, $[(k_{cat}/K_m)_o]$ and the proton dissociation constants (K_{e1} and K_{e2}) for the pH-dependence of the activity were calculated from Eq. 1 by a non-linear least-squares regression method with Kaleida Graph Version 3.5 (Synergy Software, Essex, VT, USA).

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

In this equation, $(k_{cat}/K_m)_{obs}$ and [H] mean the k_{cat}/K_m value observed and the proton concentration, respectively, at the specified pH.

Spectrophotometric Analysis of the Thermolysin-catalysed Hydrolysis of ZDFM—Hydrolysis of ZDFM catalysed by thermolysin was measured by following the decrease in absorbance at 224 nm (*12*). The amount of ZDFM hydrolysed was evaluated using the molar absorption difference due to hydrolysis, $\Delta\varepsilon_{224} = -493\ M^{-1}\ cm^{-1}$, at 25°C (*12*). The reaction was carried out with thermolysin at 0.16 μM in 40 mM Tris–HCl (pH 7.5) buffer containing 10 mM $CaCl_2$ at 25°C. The kinetic parameters, k_{cat} and K_m, were determined based on the Michaelis–Menten equation using the non-linear least-squares methods (*43*).

Thermal Inactivation of Thermolysin—Thermolysin (0.1 μM) was dissolved in 20 mM sodium acetate (pH 5.3) buffer containing 5 mM $CaCl_2$, 0.5%(v/v) 2-propanol and 63.5 mM NaCl, and was incubated at 25°C for 15 min. Then, it was incubated at a specified temperature for 30 min and at 25°C for 15 min. The remaining activity of thermolysin in casein hydrolysis was determined according to the methods described previously (*44*). 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. 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 unit of activity is defined as the amount of enzyme activity needed to liberate a quantity of acid-soluble peptide corresponding to an increase in A_{275} of 0.0074 (A_{275} of 1 μg of tyrosine)/min.

RESULTS

Production of Mutant Thermolysins—Mutant thermolysins were produced in the *E. coli* expression system (*41*) as described in the section of 'MATERIALS AND METHODS'. Starting from 350 ml of culture supernatants, 1.6–6.6 mg purified enzymes were recovered, which was comparable to the preparation of the wild-type thermolysin (1.8 mg from 290 ml of culture supernatant) (*41*). No appreciable changes were observed in UV spectra between the wild-type and mutant thermolysins (data not shown), suggesting that all mutant thermolysins did not suffer from any drastic structural changes. We then characterized the activities and thermal stabilities of the mutant thermolysins in the hydrolysis of FAGLA and ZDFM.

pH-dependence of FAGLA-hydrolyzing Activity—The pH-dependence of the specificity constant (k_{cat}/K_m) of the thermolysin-catalysed hydrolysis of FAGLA at 25°C is shown in Fig. 2. The kinetic parameters, the intrinsic k_{cat}/K_m, $[(k_{cat}/K_m)_o]$ and the pK_a values are summarized in Table 1. All plots showed bell-shaped curves with the optimal pH of 6.4–7.0. The alkaline pK_a (pK_{e2}) of Q225A was 8.48 ± 0.04, being 0.42 ± 0.07 units higher than that of the wild-type enzyme. The acidic pK_a (pK_{e1}) of Q225A and the acidic and alkaline pK_a's of the other eight mutant enzymes were almost identical with those of the wild-type enzyme. The relative $(k_{cat}/K_m)_o$ values of each mutant enzyme to the wild-type enzyme were almost identical, which were in the range of 0.7–1.1.

Salt-induced Activation in the Hydrolysis of FAGLA—Table 2 shows the k_{cat}/K_m values at 4 M NaCl and 0 M NaCl in the hydrolysis of FAGLA and the degrees of the activation, which are defined as the ratios of the k_{cat}/K_m value at 4 M NaCl to that at 0 M NaCl. The degree of the activation of the wild-type enzyme was 14, but those of Q225A and Q225V were 10 and 19, corresponding to the relative degrees of the activation of 0.7 and 1.4 to the wild-type enzyme, respectively. In contrast, the relative degrees of the other seven mutant enzymes were in the range of 0.9–1.1, suggesting that the mutation of Gln225 to Ala and Val resulted in significant changes in the degree of the NaCl-induced activation.

Kinetic Parameters in the Hydrolysis of ZDFM—ZDFM is the substrate to which the Michaelis–Menten treatment is applicable to determine k_{cat} and K_m separately. The dependence of the initial rate of the thermolysin-catalysed hydrolysis of ZDFM on the ZDFM concentration at pH 7.5, at 25°C is shown in Fig. 3. All plots showed the saturated profile of the Michaelis–Menten kinetics, and the k_{cat} and K_m values were determined (Table 3). The relative reaction rates of Q225A and Q225V to that of the wild-type enzyme were 0.3 and 0.5,

respectively (Fig. 3). The relative K_m values of Q225A and Q225V were 0.6 and 0.5, respectively, while the relative k_{cat} values of them were 0.3. Thus, the decrease in the activity of Q225A and Q225V can be ascribed to the decrease in the k_{cat} values by the mutation. On the other hand, the kinetic parameters of the other seven mutant enzymes did not change significantly, and their relative K_m and k_{cat} values to the wild-type enzyme were in the range of 0.6–1.4 and 0.7–1.0, respectively.

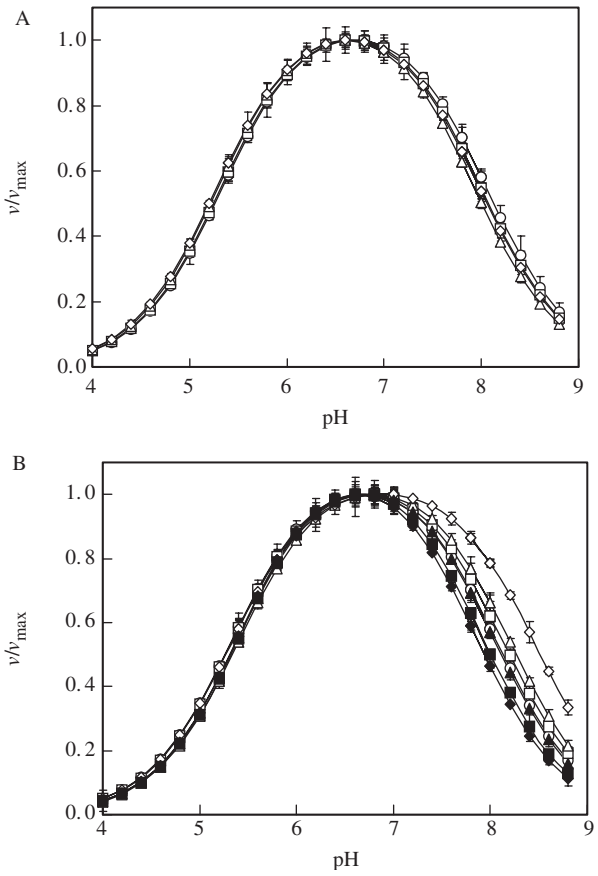

Fig. 2. **Effect of pH on the thermolysin-catalysed hydrolysis of FAGLA.** The reaction was carried out at the enzyme concentration of 0.08 μM at 25°C. The v_{max} value is the reaction rate at the optimal pH. Symbols for the enzymes: (A) the wild-type thermolysin, open circle; Q128K, open square; Q128E, open triangle and Q128A, open diamond. (B) The wild-type thermolysin, open circle; Q225K, open square; Q225R, filled square; Q225E, open triangle; Q225D, filled triangle; Q225A, open diamond and Q225V, filled diamond. Error bars indicate the SD values.

Thermal Stability—The relative activity of heat-inactivated thermolysin for casein-hydrolysis is shown in Fig. 4, and the temperature required to reduce initial activity by 50% in 30 min, T_{50}, is summarized in Table 4. All plots showed the similar sigmoid curves. The ΔT_{50} values of the mutant enzymes compared with the wild-type enzyme were in the range of (–4.8) to 4.3, and did not show any significant changes. This suggests that the mutations of Gln128 and Gln225 do not significantly change the thermal stability. However, it should be noted that there are interesting features: T_{50} decreased slightly by the replacement of Gln225 with the positively charged amino acid (Lys or Arg), while T_{50} increased by the replacement with negatively charged or non-charged residue. The effect of the replacement of Gln128 seems to be inverted from that of Gln225.

DISCUSSION

Insights into Mechanism of Changes in Thermolysin Activity by the Mutation of Gln225—In this study, the replacement of the uncharged polar amino acid residue Gln225 at the surface of thermolysin by a hydrophobic aliphatic amino acid (Ala or Val), but not a positively charged (Lys or Arg) or a negatively charged (Glu or Asp) amino acid, resulted in multiple changes in activity. The changes include the pK_{e2} value (Fig. 2, Table 1) and the degree of the activation by NaCl (Table 2) in the hydrolysis of FAGLA and ZDFM (Fig. 3, Table 3). The replacement of another surface residue Gln128 by Ala, Lys and Glu gave no significant changes. There were no significant changes in thermal stability of all mutant enzymes (Fig. 4, Table 4). Although the crystallographic data of the mutant enzymes have not been available, we speculate that the mutation-induced changes in the electrostatic environment through the conformational change in the α-helix 226–245, which contains the catalytically important residue His231.

In the following, we discuss the pK_{e2} shift of Q225A. Generally, pK_e shift is caused by a change in electrostatic environment around ionizable groups in the catalytic

Table 1. **pK_a values and intrinsic k_{cat}/K_m [$(k_{cat}/K_m)_o$] of the wild-type thermolysin and the mutant enzymes in the hydrolysis of FAGLA at 25°C.**

Thermolysin	Acidic pK_a	Alkaline pK_a	$(k_{cat}/K_m)_o \times 10^{-4}$ ($M^{-1}s^{-1}$)
Wild-type	5.33 ± 0.03 (0.00)	8.06 ± 0.03 (0.00)	4.22 ± 0.17 (1.0)
Q128K	5.32 ± 0.03 (–0.01)	8.00 ± 0.03 (–0.06)	3.27 ± 0.09 (0.8)
Q128E	5.29 ± 0.04 (–0.04)	7.93 ± 0.04 (–0.13)	3.59 ± 0.07 (0.9)
Q128A	5.27 ± 0.04 (–0.06)	7.99 ± 0.04 (–0.07)	2.80 ± 0.06 (0.7)
Q225K	5.33 ± 0.03 (0.00)	8.13 ± 0.03 (+0.07)	3.29 ± 0.06 (0.8)
Q225R	5.41 ± 0.04 (+0.08)	7.91 ± 0.04 (–0.15)	4.00 ± 0.05 (1.0)
Q225E	5.40 ± 0.05 (+0.07)	8.20 ± 0.05 (+0.14)	4.43 ± 0.19 (1.1)
Q225D	5.39 ± 0.04 (+0.06)	8.03 ± 0.04 (–0.03)	3.57 ± 0.11 (0.9)
Q225A	5.31 ± 0.03 (–0.02)	8.48 ± 0.04 (+0.42)	3.95 ± 0.04 (0.9)
Q225V	5.40 ± 0.05 (+0.07)	7.85 ± 0.05 (–0.21)	3.22 ± 0.08 (0.8)

Note: The average of triplicate determination with the SD value is shown. Numbers in parentheses indicate ΔpK_a compared to the wild-type thermolysin and the $(k_{cat}/K_m)_o$ relative to the wild-type thermolysin.

Table 2. **Degree of salt-induced activation of the wild-type thermolysin and the mutant enzymes in the hydrolysis of FAGLA at 25°C.**

Thermolysin	$k_{cat}/K_m \times 10^{-4}$ ($M^{-1} s^{-1}$)		(B/A)
	M NaCl (A)	4 M NaCl (B)	
Wild-type	2.42 ± 0.09 (1.0)	34.5 ± 6.2 (1.0)	14 (1.0)
Q128K	2.10 ± 0.10 (0.9)	26.2 ± 1.5 (0.8)	12 (0.9)
Q128E	2.48 ± 0.07 (1.0)	31.7 ± 5.5 (0.9)	13 (0.9)
Q128A	2.35 ± 0.20 (1.0)	29.8 ± 2.5 (0.9)	13 (0.9)
Q225K	2.35 ± 0.20 (1.0)	29.8 ± 2.5 (0.9)	13 (0.9)
Q225R	2.34 ± 0.23 (1.0)	35.5 ± 13.5 (1.0)	15 (1.1)
Q225E	2.41 ± 0.10 (1.0)	31.0 ± 2.4 (0.9)	13 (0.9)
Q225D	2.73 ± 0.26 (1.1)	42.3 ± 2.0 (1.2)	16 (1.1)
Q225A	3.70 ± 0.23 (1.5)	37.4 ± 6.6 (1.1)	10 (0.7)
Q225V	1.64 ± 0.18 (0.7)	30.3 ± 12.2 (0.9)	19 (1.4)

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

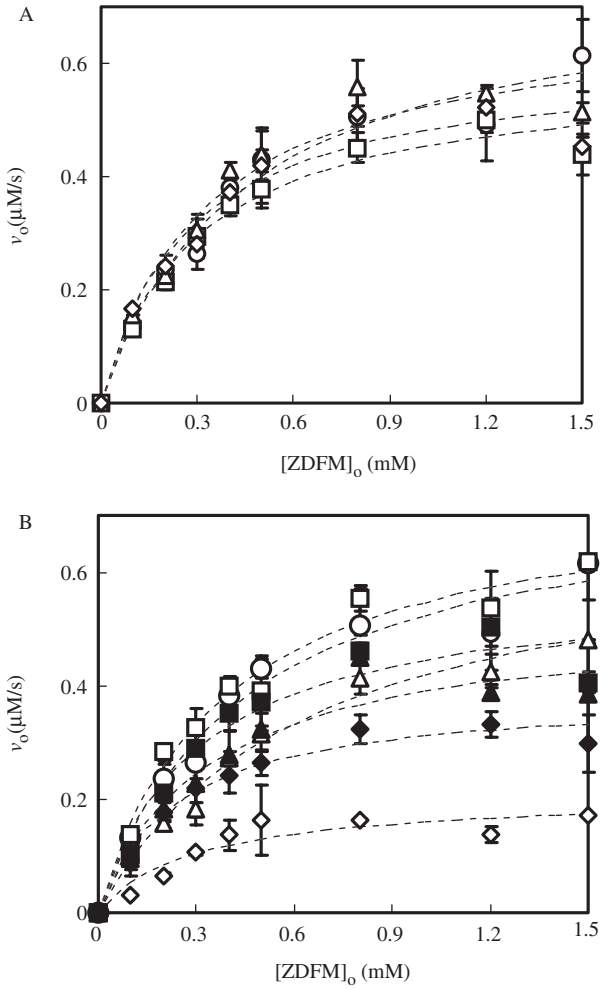

Fig. 3. **Dependence on the substrate concentration of the reaction rate of the thermolysin-catalysed hydrolysis of ZDFM.** The reaction was carried out at the enzyme concentration of 0.16 μM at 25°C. Symbols for the enzymes: (A) the wild-type thermolysin, open circle; Q128K, open square; Q128E, open triangle and Q128A, open diamond. (B) The wild-type thermolysin, open circle; Q225K, open square; Q225R, filled square; Q225E, open triangle; Q225D, filled triangle; Q225A, open diamond and Q225V, filled diamond. Error bars indicate the SD values.

mechanism. His231 has been ascribed for the ionizable group for pK_{e2} (*32*, *33*). Asp226 is the residue located between Gln225 and His231 and is thought to hold His231 by the salt bridge between either OD1 and OD2 of Asp226 and ND1 of His231 (*37*, *45*, *46*) (Fig. 1B). We speculate that the pK_{e2} shift observed in Q225A results from that the shift of Asp226 toward His231 may inhibit the deprotonation of His231. We have previously reported that the pK_{e1} value in the bell-shaped pH-dependence profile of the FAGLA-hydrolyzing activity (k_{cat}/K_m) of thermolysin is shifted from 5.4 to 6.7 by the addition of 4 M NaCl (*19*). Although the pK_{e2} shift observed in Q225A (from 8.06 to 8.48) was relatively small, it is noteworthy that a pK_e shift can occur even by a single substitution of a surface residue that locates as far as 15.8 Å from the active site zinc ion. It is surprising that the mutational effects of Gln225 depended on the species of the hydrophobic residues introduced: the pK_{e2} shift occurred in Q225A but not Q225V, and Q225A and Q225V gave the opposite effects on the activation on NaCl. We speculate that the difference in the mutational effects may result from the difference in sizes of Ala and Val.

It should also be noted that the k_{cat}/K_m values of Q225A were the highest in the hydrolysis of FAGLA (Table 2) and the lowest in the hydrolysis of ZDFM (Table 3) among the mutant thermolysins in the absence of NaCl. The similar substrate preference was observed with the mutant thermolysin N112D with the pK_{e1} value of 5.7, higher by 0.4 units than the wild-type thermolysin (*31*). The k_{cat}/K_m values of N112D in the hydrolysis of FAGLA and ZDFM were 64 and 0.23% of those of the wild-type thermolysin (*31*). We speculate that the replacement of Gln225 by Ala induced another effect on the active site although the mechanism is not clear.

Site-directed Mutagenesis of Surface Residues of Thermolysin—It was previously reported that introduction of surface charges by site-directed mutagenesis of TLP-ste gave a long-range electrostatic interaction to the active site (*47*). Our results demonstrated that introduction of hydrophobic amino acid residues at the surface of thermolysin, and possibly TLP-ste, may induce changes in the electrostatic environment in the active site without considerably decreasing its activity. This seems to contradict a general concept that hydrophobic residues

Table 3. **Kinetic parameters of the wild-type thermolysin and the mutant enzymes in the hydrolysis of ZDFM at 25°C.**

Thermolysin	K_m (mM)	k_{cat} (s^{-1})	$k_{cat}/K_m \times 10^{-4}$ (M^{-1} s^{-1})
Wild-type	0.45 ± 0.09 (1.0)	4.7 ± 0.6 (1.0)	1.04 ± 0.13 (1.0)
Q128K	0.30 ± 0.05 (0.7)	3.7 ± 0.2 (0.8)	1.23 ± 0.08 (1.2)
Q128E	0.33 ± 0.08 (0.7)	4.3 ± 0.4 (0.9)	1.30 ± 0.11 (1.3)
Q128A	0.28 ± 0.07 (0.6)	3.8 ± 0.3 (0.8)	1.36 ± 0.11 (1.3)
Q225K	0.39 ± 0.06 (0.9)	4.7 ± 0.3 (1.0)	1.21 ± 0.08 (1.2)
Q225R	0.30 ± 0.09 (0.7)	3.6 ± 0.4 (0.8)	1.20 ± 0.16 (1.2)
Q225E	0.63 ± 0.11 (1.4)	4.3 ± 0.3 (0.9)	0.68 ± 0.13 (0.7)
Q225D	0.34 ± 0.10 (0.8)	3.3 ± 0.3 (0.7)	0.97 ± 0.10 (0.9)
Q225A	0.29 ± 0.12 (0.6)	1.3 ± 0.2 (0.3)	0.45 ± 0.06 (0.4)
Q225V	0.24 ± 0.05 (0.5)	1.6 ± 0.2 (0.3)	0.67 ± 0.04 (0.6)

Note: The average of triplicate determination with the SD value is shown. Numbers in parentheses indicate values relative to the wild-type thermolysin.

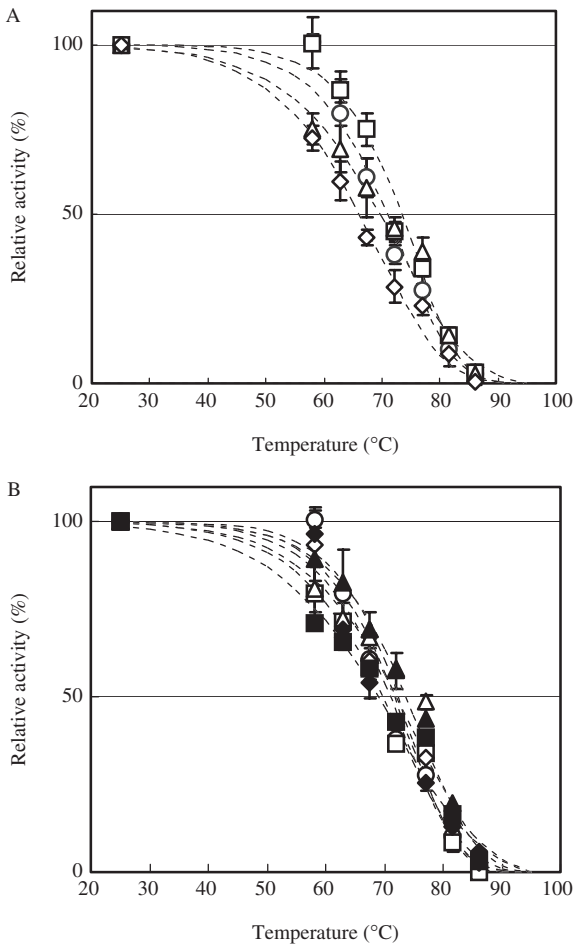

Fig. 4. **Thermal stability of the wild-type thermolysin and the mutant enzymes.** The relative activity of thermolysin for casein-hydrolysis is defined as the ratio of the activity (units/ml) with the 30-min incubation at the temperature indicated to that at 25°C (the wild-type thermolysin, 30.4 units/ml; Q128K, 24.0 units/ml; Q128E, 34.0 units/ml; Q128A, 32.2 units/ml; Q225K, 31.2 units/ml; Q225R, 24.7 units/ml; Q225E, 35.4 units/ml; Q225D, 35.3 units/ml; Q225A, 39.0 units/ml; Q225V, 17.2 units/ml). Symbols for the enzymes: (A) the wild-type thermolysin, open circle; Q128K, open square; Q128E, open triangle and Q128A, open diamond. (B) The wild-type thermolysin, open circle; Q225K, open square; Q225R, filled square; Q225E, open triangle; Q225D, filled triangle; Q225A, open diamond; Q225V, filled diamond. Error bars indicate the SD values.

Table 4. **Thermal stability of the wild-type thermolysin and the mutant enzymes.**

Thermolysin	T_{50}[a] (°C)
Wild-type	70.9 ± 0.5
Q128K	73.2 ± 0.5 (+2.3)
Q128E	69.6 ± 0.9 (–1.3)
Q128A	66.1 ± 0.8 (–4.8)
Q225K	69.7 ± 0.7 (–1.2)
Q225R	70.1 ± 1.1 (–0.8)
Q225E	72.0 ± 1.1 (+1.1)
Q225D	75.2 ± 0.4 (+4.3)
Q225A	71.6 ± 1.2 (+0.7)
Q225V	73.4 ± 1.8 (+2.5)

[a] T_{50} is the temperature required to reduce initial activity by 50% in 30 min. The average of triplicate determination with the SD value is shown. Numbers in parentheses indicate ΔT_{50} compared to the wild-type thermolysin.

in proteins are not favourable at solvent-exposed sites. However, distinct conclusions have been reported in some proteins. In α-amylase from *Bacillus licheniformis*, a surface-residue triple mutant H133V/N190F/A209V unfolded 27 times more slowly than the wild-type enzyme (*48*). In cold-shock protein CspB from *B. subtilis*, three surface residues Phe15, Phe17 and Phe27 played important roles in stabilities as well as activities (*49*). In the all-β-sheet protein named cluster determinant molecular domain 1 (CD2.d1), introduction of hydrophobic residues at various positions of the surface stabilized the protein (*50*). These reports suggest that introduction of hydrophobic residues at the surface does not always make the protein thermodynamically unfavourable.

It is generally known that the results of mutagenesis are unpredictable. Indeed, several mutant thermolysins exhibiting the improved activity or thermal stability were isolated rather unexpectedly (*26*, *27*, *35*). The results in this study, together with our previous findings on the effects of solvent composition on thermolysin activity, the remarkable activation by neutral salts (*9*, *12*, *18–20*) and the inhibition by alcohols (*19*, *22*), suggest that site-directed mutagenesis of surface residues of thermolysin, including introduction of hydrophobic residues, should be an attractive strategy not only to understand the mechanism of the effects of solvent composition but also to improve its activity and stability.

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