Remarkable activity enhancement of thermolysin mutants

Shun-ichi Kidokoro[a,*], Yoichiro Miki[a], Kimiko Endo[a], Akiyoshi Wada[a], Hiromasa Nagao[b], Toshio Miyake[b], Atsuo Aoyama[b], Takashi Yoneya[b], Kenichi Kai[b], Seigo Ooe[b]

[a]*Sagami Chemical Research Center, Nishiohnuma 4-4-1, Sagamihara, Kanagawa 229, Japan*
[b]*TOSOH Corp. Biotechnology Research Laboratory, Hayakawa 2743-1, Ayase, Kanagawa 252, Japan*

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Abstract Most attempts to modify the properties of enzymes by amino acid substitution around the active sites have resulted in suppression of the biological activity, suggesting that the structure of natural enzymes should be almost optimized evolutionally to show the highest activity. In contrast, we found an interesting site of a well-known metalloendopeptidase, thermolysin (EC. 3.4.24.4), where almost all the amino acid replacement causes a remarkable increase in the hydrolytic activity. Negative correlation between the activity and the thermal stability was observed. The flexibility around the substrate binding site is suggested to be a key to the correlation. Nature may have selected the amino acid at this site, which suppresses the flexibility of the molecule, to get the highest thermal stability at the expense of the activity.

Key words: Enzyme stability; Activity; Design principle; Amino acid mutation

1. Introduction

In recent protein engineering study, most attempts to modify the stability and/or activity by amino acid substitution near the active sites have resulted in suppression of the biological activity, while the thermal stability of proteins is able to be enhanced by introducing or reinforcing several phisico-chemical intramolecular interactions, such as hydrogen bonding, S–S bridge etc. It suggests that the structure of natural enzymes around the active sites should be almost optimized evolutionally to show the highest activity rather than the highest stability.

In order to establish a new strategy for enhancing enzyme activity, we have started the protein engineering study of thermolysin. This is one of the best studied enzymes, and widely used by laboratories and industry because of its narrow specificity for hydrophobic residues and high thermal stability. In engineering, this enzyme is used as a useful catalyst for industrial production of the artificial sweetener, aspartame [1]. The amino acid sequence [2,3] and the three-dimensional (3D) structure with several inhibitors [4,5] have been determined.

By analyzing the 3D structure, we were aware that the 119th residue was an interesting location for amino acid substitution for two reasons. First, the site was considered to be highly effective in producing the electrostatic field at an activesite of the enzyme, the 143rd glutamate, judging from the electrostatic calculation, indicating that the change of electric charge at this site may affect the activity [6]. Secondly, the 119th glutamine-was estimated to play a role in stabilizing the structureby forming a hydrogen bond with the hydroxyl group of the 103rd serine, as already proposed for the neutral protease from *Bacillus stearothermophilus* [7]. The breakage of the hydrogen bond may affect the activity and the thermal stability with changing the flexibility around the substrate binding site.

It should be noted that the cDNA sequencing [8] and our re-examination of direct amino acid sequencing of the enzyme [9] have corrected the known sequence by two residues, and that the 119th happens to correspond to one of them. The corrected sequence is identical with that of a neutral protease from *Bacillus stearothermophilus* [7].

2. Materials and methods

2.1. Mutants of thermolysin

The site-directed mutagenesis at the 119th position of thermolysin was performed by overlap extension method [10] using the polymerase chain reaction. The mismatched primer and its complement sequence were synthesized and used for inside primers, which anneal to the same segment of *npr* M [7] gene. The mismatches lead to random sequence alteration in the 119th amino acid residue of thermolysin. Expression plasmid pUBTZ2 which is a shuttle vector between *E. coli* and *B. subtilis*, was constructed from pMK4[11] by ligating with pUB110 [12]. The amplified DNA fragment containing random mutation was digested with *Bam*HI and *Sph*I, and replaced the same region of the native *npr* M gene in pUBTZ2. The mutant thermolysins were expressed in *B. subtilis* MT-2 by cultivating the transformants with LB medium containing 20 μg/ml kanamycin. The mutant thermolysins were recovered by ammonium sulfate precipitation of culture supernatant and further purified by hydrophobic chromatography and gel permeation chromatography. The amino acid at the 119th site was checked by sequencing the plasmid DNAs. The purity and the isoelectric point were determined by gel isoelectric focusing using Phast System (Pharmacia, Sweden). The purity was above 95% for all the samples except the tryptophan mutant. The p*I* values of the three mutants, Q119K, Q119R and Q119M, are 5.3, while that of other mutants and wild type is 5.0. The difference of p*I* is considered to be derived from the change of one electric charge of the mutant judging from the p*I* calculation with the amino acid composition.

All the mutants but cystein at this position were synthesized and purified. Because the purity of the tryptophan mutant was less than 90% and was not obtained in adequate quantity for the physicochemical measurement, the mutant was not further investigated in this study.

2.2. CD spectrum and DSC measurement

Circular dichloism (CD) spectrum was measured with J-500 spectrometer (Jasco, Japan), equipped with temeprature controlled water circulator at pH 8. 3 50 mM HEPES buffer containing 10 mM $CaCl_2$ and 100 μM $ZnSO_4$ at 25°C. The near and far UV CD spectra of all the 17 mutants coincide with those of the wild type enzyme (data not

*Corresponding author. Sagami Chemical Research Center, Nishiohnuma 4-4-1, Sagamihara, Kanagawa 229, Japan. Fax: (81) (427) 49 7631.

Abbreviations: 3D, three-dimensional; CD, circular dichroism; DSC, differential scaning calorimetry; FAGLA, *N*-(3-[2-furyl]acryloyl)-glycyl-L-leucine amide; FADFM, *N*-(3-[2-furyl]acryloyl)-aspartyl-L-phenylalanine methylester.

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shown),suggesting that the secondary and tertiary structures of the mutants are the same as the wild type.

The thermal stability was evaluated by the peak temperature of the apparent heat absorption curve measured with a scanning calorimeter, MCS (MicroCalCo., USA) at pH 8.3 in 50 mM HEPES buffer containing 10 mM $CaCl_2$ and 100 μM $ZnSO_4$. The protein concentration was determined by photometry with the extinction coefficient of 1.67×10^4 OD/g/ml. The concentration is 0.2–0.5 mg/ml and the scanning rate is 1 K/min.

2.3. Activity measurement

The hydrolytic activities of wild type and the mutants were evaluated by measuring hydrolysis of *N*-(3-[2-furyl]acryloyl)-glycyl-L-leucine amide (FAGLA) and *N*-(3-[2-furyl]acryloyl)-aspartyl-L-phenylalanine methylester (FADFM).

Theoretical curve of an integral Michaelis–Menten equation [13] fitted well to every experimental piece of data within experimental error (10^{-3} optical density). The decrease in absorbance at 345 nm was monitored by a spectrophotometer UB-35 (JASCO, Japan) connected to a personal computer PC9801 (NEC, Japan) for data acquisition and thermostat circulator EL-15 (Taitech, Japan). The stored data were fitted with an integral Michaelis–Menten equation [13] by a non-linear least-squares method [15] in order to determine k_{cat} and K_M. All measurements were done under a solution condition: 200 mM Tris/maleate buffer with 10 mM $CaCl_2$ at 37.0°C. The concentration of the substrate was about 1–2 mM and that of the enzyme was about 1nM. The protein concentration was determined by photometry with the extinction coefficient of 1. 67×10^4 OD/g/ml. The computer program was described by us in BASIC on PC9801 and FORTRAN with a program package SALS [15] on a workstation, Titan 750 (Kubota Computer, Japan). FAGLA was purchased from Sigma (USA) and FADFM was synthesized by us with *N*-(3-[2-furyl]acryloyl)-aspartic acid and phenylalanine amide by a modified method of Oyama et al. [1]. The molar extinction change of the substrate according to the hydrolysis was determined as 7.03×10^{-2} M^{-1} (FADFM) by quantitative analysis of the concentration change of amino group with fluorescamine method [16] using a fluorescence spectrophotometer, FP-777 (Jasco, Japan). The substrate FAGLA has low affinity (high K_M value) for thermolysin, therefore only combined k_{cat}/K_M values could be obtained.

3. Results and discussion

The hydrolytic activity of the mutants depending on pH is presented in Fig. 2. The most activated mutant, Q119E, has five times higher hydrolytic activity than wild type. It should be noted that the hydrolytic activities of almost all these mutants exceed that of wild type, indicating that the amino acid at this position is naturally selected to show the lowest activity.

We found low K_M values of another substrate, FADFM, that enabled us to determine k_{cat} and K_M values separately. It is remarkable that FADFM becomes the 'good' substrate for thermolysin because the acidic residue at the P1 site was said not to be appropriate for the enzyme activity [14], and little hydrolytic study of this enzyme has been done with this kind of substrate. The FADFM hydrolytic activity of several mutants and its correlation to the FAGLA hydrolytic activity is shown in Table 1. The k_{cat}/K_M values of FADFM correlate completely with those of FAGLA. These values are strongly correlated with K_M^{-1} values (approximately considered to be the association constant of the substrate), but not with k_{cat}

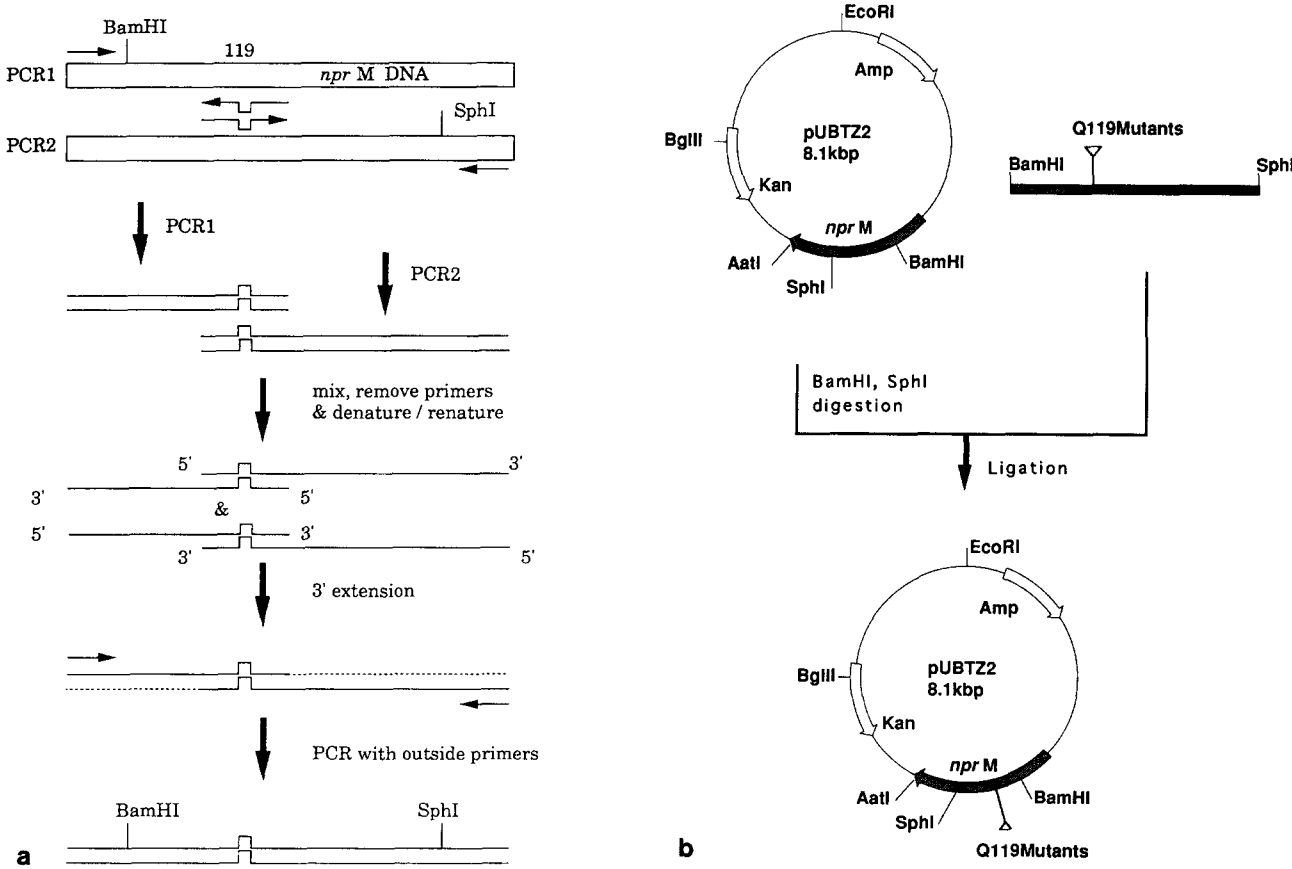

Fig. 1. Schematic diagram of site-directed mutagenesis by overlap extension and construction of the expression plasmid pUBTZ2. (a) The mismatched primer and its complement sequence. The mismatches lead to random sequence alteration in the 119th amino acid residue of thermolysin. (b) Expression plasmid pUBTZ2. The amplified DNA fragment containing random mutation was digested with *Bam*HI and *Sph*I, and replaced the same region of the native *npr* M gene in pUBTZ2.

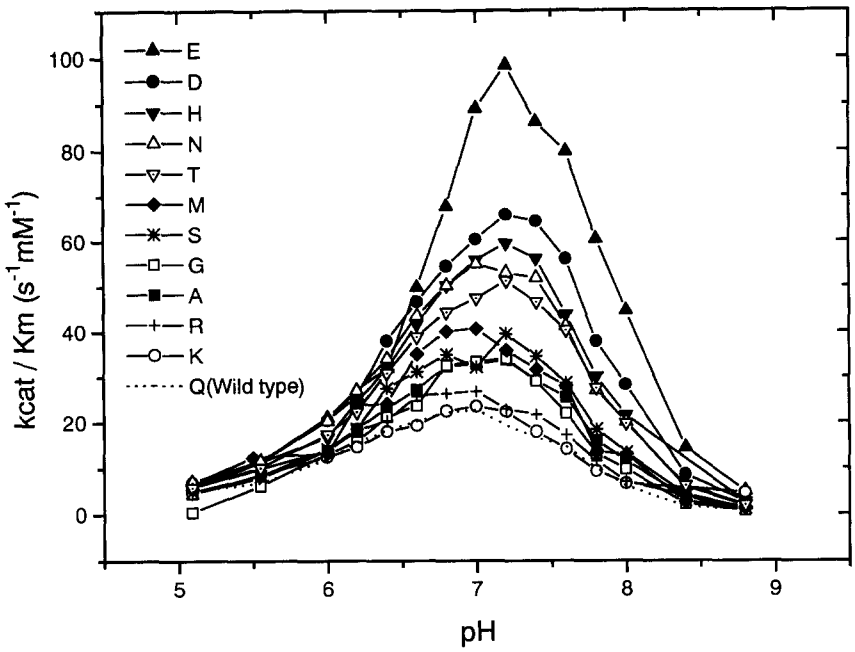

Fig. 2. The pH dependence of the hydrolytic activity of thermolysin and its mutants at the 119th position under a solution condition: 200 mM Tris/maleate buffer with 10 mM $CaCl_2$ at 37.0°C.

values. This clearly shows that the mutation increases the affinity of the substrate, so that it enhances k_{cat}/K_M for FADFM.

Judging from the three-dimensional structure of thermolysin, the side chain of the 119th glutamine is a proton donor of hydrogen bond to the side chain of the 103rd serine. The asparagine residue at this position could not be a proton donor because the distance between the 119th Cα atom and the 103rd Oγ atom seems to be too long to make a hydrogen bond. As the residue locates on the enzyme surface and the solvent accessibility of this position (30%) is not low, large residues such as tyrosine, lysine or arginine might be accepted, but they could not become a proton donor for the hydrogen bond.

The strand from the 112th to 117th residues forms one side wall of the substrate binding site. The 119th residue is thought to play a role in fixing this strand to other parts by forming a hydrogen bond. By breaking the hydrogen bonding accompanying the amino acid replacement, the flexibility of the strand will increase. This may facilitate the binding of the substrate to the enzyme, and increase the enzymatic activity.

The thermal stability of the mutants at the 119th site is expected to change because of the increased flexibility. As shown in the case of wild type [17], the thermal denaturation of all the mutants is completely irreversible judging from thermal analysis with a differential scanning calorimeter (data not shown). It was proposed that the rate-limiting step for the irreversible denaturation is not the self-hydrolysis accompanying the denaturation, and that the irreversible process is well-approximated by a first order reaction [18]. We have confirmed that the thermal transition is little affected by the concentration of a known inhibitor of thermolysin, phenanthrolin, up to 10 mM where the activity is suppressed drastically. The insensitivity of the thermal transition upon the inhibitor concentration clearly shows that the hydrolytic process is not a rate-limiting step, indicating that the unfolding process itself is the rate-limiting step.

Thermal analysis with a differential scanning calorimeter shows that the thermal stability of all the mutants is lower than that of wild type (Fig. 3). This suggests that the lack of the hydrogen bond in all the mutants may cause destabilization of the three-dimensional structure by increasing the rate of the unfolding process.

As seen in Fig. 3, the activity (k_{cat}/K_M) and the thermal stability are correlated negatively. A positive correlation is expected between the activity and the flexibility around the substrate binding site because the latter may enhance the substrate affinity, and a negative correlation between the flexibility and the thermal stability is suggested [19]. These are thought to make an overall negative correlation between the activity and the stability.

We have synthesized alanine and glycine mutants at the 103rd site, S103A and S103G, where the hydrogen bond should be broken. The two mutants have 3.0 and 2.6 times higher

Table 1
FADFM hydrolytic activity of the 119th mutants

	Q (wild type)	P	G	V	D	E	R_c
k_{cat}/K_M ($s^{-1}\cdot mM^{-1}$)	25.2	27.7	28.7	31.6	46.9	58.0	98%
k_{cat} (s^{-1})	28.9	26.2	19.5	15.1	17.8	28.2	−1%
$1/K_M$ (mM^{-1})	0.87	1.05	1.48	2.09	2.64	2.06	73%

The hydrolytic activity of FADFM was measured at pH 6.4 by the same method as FAGLA in Fig. 2. R_c is the correlation coefficient with the $k$cat/K_M values of FAGLA.

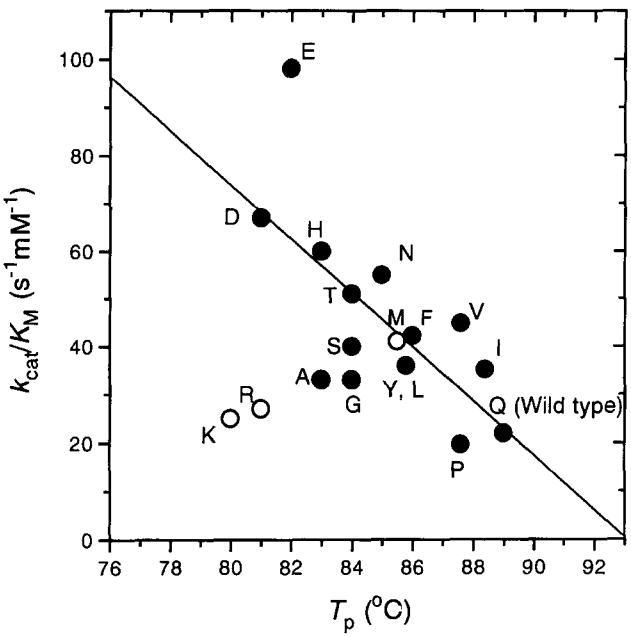

Fig. 3. Negative correlation between thermal stability and activity of mutants at the 119th residue. The maximum value of kinetic parameters k_{cat}/K_M is plotted against the apparent temperature of thermal denaturation. The 119th amino acids are indicated by one-letter representation. The three mutants, Q119K, Q119R, and Q119M are plotted with open circles, which show the large positive p*I* shifts monitored by gel isoelectric focusing as described in the text. The straight line is determined by least-squares fitting (correlation coefficient is −0.69) with all values but K, R. and M. The thermal stability was evaluated by the peak temperature of the apparent heat absorption curve.

hydrolytic activity than wild type, respectively, and the T_p values are found to be remarkably lower as seen in the 119th mutants. The same effect observed at the 103rd site supports that the breakage of the hydrogen bond is the main cause of the negative correlation between the activity and the stability.

In two mutants, Q119K and Q119R, the positive charge is introduced as judged from the p*I* shift. In this case, the k_{cat}/K_M is lower than the estimated values from the negative correlation between k_{cat}/K_M and T_p, thus indicating that the positive charge affects the activity through electrostatic interaction, although details of the mechanism are not known.

On the other hand, the plotted point of the methionine mutant, Q119M, which shows the same p*I* shift as these two mutants, lies in the straight line in Fig. 3. The mutated methionine itself brings no true charge on the 119th location where electric charge should have substantial effect on the electrostatic field at an active site, the 143rd glutamate. It may be the reason for the no deviation from the straight line in contrast to the case of Q119K and Q119R. The mechanism of the p*I* shift of Q119M, however, has not been identified.

No p*I* shift of the aspartate and glutamate mutants, Q119D and Q119E, indicates that both residues are neutralized around pH 5. 0. There is a possibility that both residues are negatively charged in the neutral pH region where the activity is evaluated. The rather large deviation of the glutamate mutant from the straight line in Fig. 3 may suggest that the negative charge at this position enhances the activity, while the positive charge suppresses it as mentioned above.

The glutamine at the 119th site is conserved through evolution as seen in the sequence alignment among the homologous neutral proteases [18], not only from thermophilic bacteria but also from mesophilic bacteria, indicating that the glutamine was fixed at an early stage of evolution, and has been conserved up to the present stage. Concerning the activity, this site is not optimized at all. This is a clear example to show that natural enzyme is not always optimized in their activity.

Evolutional optimization of enzymes by nature should be recognized in multidimensional space with several property axes, such as activity, stability and specificity. The most simple case of multidimensional space is seen in Fig. 3. Generally, these properties on each axis may have some correlations as shown in the figure. In an evolutional stage, one amino acid should be selected from the multidimensional space at a particular site. In the 119th residue of thermolysin, nature has selected the glutamine in order to obtain the highest thermal stability at the expense of the lowest activity. Even the lowest value may be sufficient to meet the biological requirement in this case.

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