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Engineering of the pH-Dependence of Thermolysin Activity as Examined by Site-Directed Mutagenesis of Asn112 Located at the Active Site of Thermolysin

Masayuki Kusano, Kiyoshi Yasukawa, Yasuhiko Hashida and Kuniyo Inouye*
Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502

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Asn112 is located at the active site of thermolysin, 5–8 Å from the catalytic Zn^{2+} and catalytic residues Glu143 and His231. When Asn112 was replaced with Ala, Asp, Glu, Lys, His, and Arg by site-directed mutagenesis, the mutant enzymes N112D and N112E, in which Asn112 is replaced with Asp and Glu, respectively, were secreted as an active form into *Escherichia coli* culture medium, while the other four were not. In the hydrolysis of a neutral substrate *N*-[3-(2-furyl)acryloyl]-Gly-L-Leu amide, the k_{cat}/K_m values of N112D and N112E exhibited bell-shaped pH-dependence, as did the wild-type thermolysin (WT). The acidic pK_a of N112D was 5.7 ± 0.1, higher by 0.4 ± 0.2 units than that of WT, suggesting that the introduced negative charge suppressed the protonation of Glu143 or Zn^{2+}-OH. In the hydrolysis of a negatively charged substrate, *N*-carbobenzoxy-L-Asp-L-Phe methyl ester (ZDFM), the pH-dependence of k_{cat}/K_m of the mutants decreased with increase in pH from 5.5 to 8.5, while that of WT was bell-shaped. This difference might be explained by the electrostatic repulsion between the introduced Asp/Glu and ZDFM, suggesting that introducing ionizing residues into the active site of thermolysin might be an effective means of modifying its pH-activity profile.

Key words: metalloproteinase, pH-activity profile, purification, site-directed mutagenesis, thermolysin.

Abbreviations: FAGLA, *N*-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide; *npr*, neutral protease gene; FM, L-phenylalanine methyl ester; ZD, *N*-carbobenzoxy-L-aspartic acid; 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 catalyzes specifically the hydrolysis of peptide bonds containing hydrophobic amino acid residues (*8*, *9*). Thermolysin is widely used for peptide-bond formation through the reverse reaction of hydrolysis (*10–12*). We have evaluated the effects of solvent composition on the activity and reported remarkable activation and stabilization by high concentrations (1–4 M) of neutral salts (*9*, *13–15*), crystallographic analysis in the presence of 4 M NaCl (*16*), the activation by cobalt-substitution of the catalytic zinc ion (*17*), and inhibitory effects of alcohols (*18*, *19*). These data show that the activity depends on the dielectric constant of the reaction medium, suggesting the importance of surface charges of the molecule. Thermolysin activity (k_{cat}/K_m) in the hydrolysis of *N*-[3-(2-furyl) acryloyl]-glycyl-L-leucine amide (FAGLA), a widely used neutral substrate for thermolysin, exhibits bell-shaped pH-dependence with pK_a values of 5.0–5.6 and 7.5–8.3 (*18*). The pK_a at the acidic side (pK_{e1}) shifted from 5.4 to 6.7 with increasing NaCl concentration from 0 to 4 M (*9*). Interestingly, no such pK_a shift was observed for cobalt-substituted thermolysin (*17*). Site-directed mutagenesis of thermolysin or thermolysin-like protease has clarified the involvement of Glu143 and His231 in the catalysis (*20*, *21*) and generated the enzyme with higher activity and stability (*22–25*). The assignment of the residue responsible for pK_{e1}, however, is a matter of controversy. The Lewis acid of the catalytic zinc other than Glu143 is also supposed to be the group for pK_{e1} (*18*, *26*). Tailoring the pH-activity profile of thermolysin has not been achieved so far by site-directed mutagenesis.

The aim of this study is to modify the pH-activity profile of thermolysin by introducing charges at the active site by site-directed mutagenesis. For this purpose, we previously established the *E. coli* expression system for extracellular production of recombinant thermolysin (*27*). Single mutations were introduced at Asn112 at the active site of thermolysin. The mutants were expressed, purified, and characterized by determining their pH-activity profiles in the hydrolysis of FAGLA and *N*-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester (ZDFM), a negatively charged substrate and a precursor of a synthetic sweetener, aspartame (*13*).

MATERIALS AND METHODS

Materials—FAGLA (Lot 111K1764) was purchased from Sigma (St. Louis, MO). The concentration of FAGLA was determined spectrophotometrically using the molar

*To whom correspondence should be addressed. Tel: +81-75-753-6266, Fax: +81-75-753-6265, E-mail: inouye@kais.kyoto-u.ac.jp

absorption coefficient, ε_{345} = 766 $M^{-1}\cdot cm^{-1}$ (*13*, *28*). ZDFM was prepared as described previously (*13*). The concentration of ZDFM was determined using the molar absorption coefficient, ε_{257} = 387 $M^{-1}\cdot cm^{-1}$ (*13*).

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. pTE1 is an expression plasmid that contains a 2033-bp fragment containing the complete *npr* gene and the promoter region, as described previously (*27*). Site-directed mutagenesis was carried out using Quickchange™ Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). The nucleotide sequences of mutated thermolysin genes were verified by use of a Shimadzu DNA sequencer DSQ-2000 (Kyoto). JM109 cells were transformed with each of the resulting plasmids and cultured in L broth. Ampicillin was used at the concentration of 50 μg/ml.

SDS-PAGE—SDS-PAGE was performed in a 12.5% polyacrylamide gel under reducing conditions according to the method of Laemmli (*29*). A constant current of 40 mA was applied for 1 h. 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. The 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 a product of Daiichi Pure Chemicals (Tokyo).

Hydrolysis of Casein—The activity of supernatants toward casein hydrolysis was determined according to the methods described previously (*4*, *30*). Samples (0.5 ml) were 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 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.

Production of Mutant Enzymes—Fermentation and purification of the wild-type thermolysin and the thermolysin mutants, N112D and N112E, in which Asn112 was replaced with Asp and Glu, respectively, were performed as described previously (*27*, *31*). Active and mature thermolysin was purified to homogeneity from the supernatant of *E. coli* cells by 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-Catalyzed Hydrolysis of FAGLA—Hydrolysis of FAGLA by thermolysin was measured following the decrease in absorbance at 345 nm (*9*, *13*). The amount of FA-dipeptide amides hydrolyzed was evaluated by using the molar absorption difference due to hydrolysis, $\Delta\varepsilon_{345}$ = –310 $M^{-1}\cdot cm^{-1}$, at 25°C (*13*, *28*). The reaction was carried out in 40 mM acetate-NaOH buffer at pH 4.0–5.5, 40 mM MES buffer at pH 5.5–7.0, 40 mM HEPES buffer at pH 7.0–8.5, and TAPS buffer at pH 8.0–9.0, each 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) (*13*), because of the sparing solubility (<6 mM) of FAGLA (*13*, *28*, *32*). Under these conditions, the kinetic parameters, K_m and the molecular activity k_{cat}, cannot be determined separately. Instead, the enzyme reaction was performed under pseudo-first order conditions at pH 4.0–9.0, and the enzyme activity was evaluated by the specificity constant, k_{cat}/K_m.

Analysis of the pH-Dependence Curve of Thermolysin Activity—The pK_a values given from the pH-dependence of the k_{cat}/K_m are the values with respect to the catalytic residues located in the enzyme, but those given from the pH dependence of the k_{cat} are those with respect to the catalytic residues in the enzyme-substrate complex (*33*). Hereinafter, the acidic and alkaline pK_as obtained from the pH dependence of the k_{cat}/K_m are designated as pK_{e1} and pK_{e2}, respectively, and those obtained from the pH-dependence of the k_{cat} are as pK_{es1} and pK_{es2}. From the initial velocity, kinetic parameters [the catalytic constant (k_{cat}), Michaelis constant (K_m), specificity constant (k_{cat}/K_m)] were calculated using the Michaelis-Menten equation with Kaleida Graph ver. 3.5 (Synergy Software, Reading, PA, USA). The bell-shaped pH dependence of k_{cat}/K_m and k_{cat} was analyzed by the model of two catalytically functional ionizing groups (*33*, *34*). The intrinsic specific constant $(k_{cat}/K_m)_o$, which is independent of pH, and the proton dissociation constants (K_{e1} and K_{e2}) in the free state of the enzyme were calculated from the pH-dependence of k_{cat}/K_m using Eq. 1.

$$k_{cat}/K_m = (k_{cat}/K_m)_0/\{1 + ([H]/K_{e1}) + (K_{e2}/[H])\} \quad (1)$$

where pK_{e1} = –log K_{e1}; pK_{e2} = –log K_{e2}; pK_{es1} = –log K_{es1}; pK_{es2} = –log K_{es2}; and pK_{e1} and pK_{es1} are on the acidic side, and pK_{e2} and pK_{es2} are on the alkaline side of the bell-shaped pH-dependence of k_{cat}/K_m and k_{cat}, respectively. The intrinsic catalytic constant $(k_{cat})_o$, which is independent of pH, and the proton dissociation constants (K_{es1} and K_{es2}) of the catalytic groups in the enzyme-substrate complex were calculated from the pH dependence of k_{cat} using Eq. 2.

$$k_{cat} = (k_{cat})_o/\{1 + ([H]/K_{es1}) + (K_{es2}/[H])\} \quad (2)$$

HPLC Analysis of the Thermolysin-Catalyzed Hydrolysis of ZDFM—We have newly established a HPLC system to detect ZDFM, *N*-carbobenzoxy-L-aspartic acid (ZD), and L-phenylalanine methyl ester (FM), details of which will be published elsewhere. Briefly, the hydrolysis of ZDFM was initiated by adding 50 μl of a thermolysin solution (2–5 μM) to 950 μl of ZDFM (0.11–1.58 mM) in the reaction buffer. The reaction was carried out at 25°C in 40 mM MES buffer at pH 5.5–7.0, and 40 mM HEPES buffer at pH 7.5–8.5, each containing 10 mM $CaCl_2$. The reaction was stopped at an appropriate time by adding 200 μl of 200 mM EDTA2Na. The mixture (100 μl) was then applied to reversed-phase HPLC performed on a TSKgel ODS-80Ts column [4.6 mm (inner diameter) × 150 mm] (Tosoh, Tokyo) equilibrated with

10% acetonitrile and 0.1% TFA. A linear gradient was generated from 10 to 70% (v/v) acetonitrile at the retention time of 5 min over 25 min at a flow-rate of 1.0 ml/min. The absorption of eluates was detected at 254 nm. The substrate ZDFM and its two products, ZD and FM, were separated. The initial rate of hydrolysis was determined from the time course of the amount of ZD produced, which was evaluated from its peak area. The kinetic parameters k_{cat} and K_m were determined based on the Michaelis-Menten equation using the nonlinear least-squares methods (*35*). The HPLC apparatus, consisting of solvent delivery system CCPM, a UV monitoring system UV-8010, a computer control system PX-8010, and an integrator Chromatocorder 21, was purchased from Tosoh.

Design of Mutant Thermolysins—Figure 1A shows the structure of thermolysin based on the crystallographic analysis (*36*). The active-site cleft is clearly observed between the N- and C-terminal domains. The ionizable residues responsible for the pK_a values (pK_{e1} and pK_{e2}) on the acidic and alkaline sides of the pH-dependence of thermolysin activity are considered to be Glu143 or Zn^{2+}-OH and His231, respectively (*20*, *21*, *26*). Asn112 is in the N-terminal domain and protrudes into the active site. The distances between OD1 of Asn112 and OE1 of Glu143, NE2 of His231, and Zn^{2+} ion are 5.3, 6.5, and 7.5 Å, respectively (Fig. 1B). Asn112 was replaced with a negatively charged amino acid (either Asp or Glu), a positively charged one (Arg, Lys, or His), or an uncharged one (Ala).

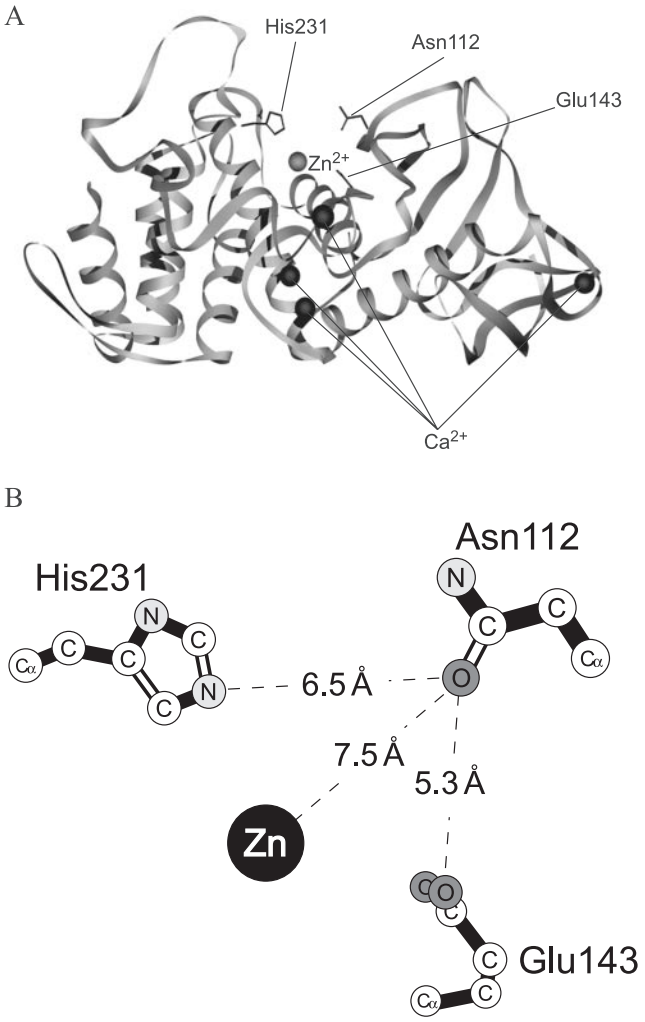

Fig. 1. **Stereochemical relationships between Asn112, Glu143, His231, and active site Zn^{2+} of thermolysin.** The overall protein structure (A) and the active site (B) of thermolysin (Protein Data Bank number 8TLN) (*36*) are shown.

RESULTS

Production of Mutant Thermolysins—Figure 2 shows SDS-PAGE of the culture supernatants of the *E. coli* cells transformed with the plasmids containing mutant thermolysin genes. The 34-kDa protein band corresponding to mature thermolysin was detected for the wild-type thermolysin, N112D, and N112E. The supernatants of *E. coli* cells expressing the wild-type thermolysin, N112D, and N112E had casein hydrolysis activity (Table 1). The expression level of the 34-kDa protein and the casein hydrolysis activity were in the order of the wild-type thermolysin > N112D > N112E. Neither the 34-kDa protein band nor the casein hydrolysis activity was detected for N112A, N112H, N112K, and N112R. The wild-type thermolysin, N112D, and N112E were

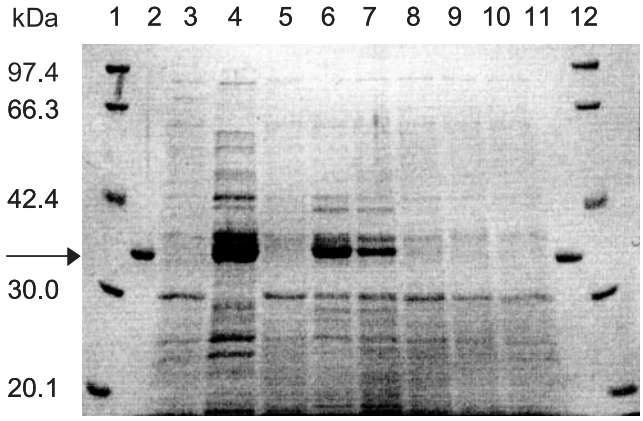

Fig. 2. **SDS-PAGE of supernatants of *E. coli* culture**. Coomassie Brilliant Blue–stained 12.5% SDS–polyacrylamide gel showing the marker proteins (lanes 1 and 12), native thermolysin from *B. thermoproteolyticus* (lanes 2 and 11), and the supernatants of *E. coli* cells transformed with pUC19 (lane 3), the pTE1 expression plasmid for wild-type thermolysin (lane 4), and the expression plasmid for the mutant thermolysin N112A (lane 5), N112D (lane 6), N112E (lane 7), N112H (lane 8), N112K (lane 9), and N112R (lane 10). The arrow indicates the band corresponding to mature thermolysin.

Table 1. **Casein hydrolysis activity of the supernatants of *E. coli* cells expressing thermolysins.**

Thermolysin	units/ml
Wild type	354
N112A	ND
N112D	62
N112E	18
N112H	ND
N112K	ND
N112R	ND

ND: not detected (<10 units/ml).

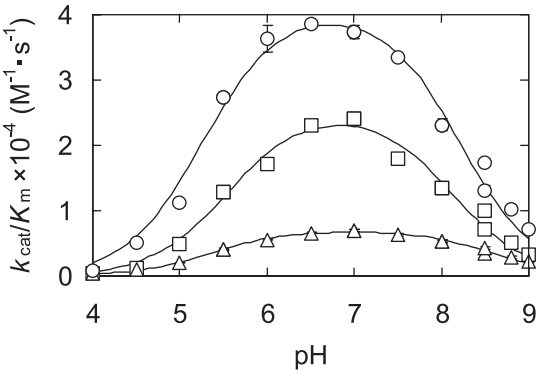

Fig. 3. **Effect of pH on the thermolysin-catalyzed hydrolysis of FAGLA.** The enzyme activity is shown by the specificity constant (k_{cat}/K_m). The reaction was carried out as described in "MATERIALS AND METHODS" at the enzyme concentration of 0.1 μM at 25°C. Symbols for the enzymes: the wild-type thermolysin, circles; N112D, squares; and N112E, triangles.

Table 2. **pK_a values of thermolysins in the hydrolysis of FAGLA at 25°C.**

Thermolysin	Acidic pK_a (pK_{e1})	Alkaline pK_a (pK_{e2})
Native	5.3 ± 0.1	8.2 ± 0.1
N112D	5.7 ± 0.1	8.1 ± 0.0
N112E	5.4 ± 0.1	8.4 ± 0.1

purified to homogeneity by sequential column-chromatography procedures.

Shift in the pK_a Values of the Thermolysin-Catalyzed Hydrolysis of FAGLA by Mutation—The pH-dependence of the specificity constant (k_{cat}/K_m) of the thermolysin-catalyzed hydrolysis of FAGLA at 25°C is shown in Fig. 3, and the pK_a values are summarized in Table 2. In the wild-type thermolysin, N112D, and N112E, the pH-dependence exhibited a bell-shaped curve with the optimum pH around 7. The k_{cat}/K_m values at pH 7.0 of N112D and N112E were 64% and 19% of that [$(3.7 \pm 0.1) \times 10^4$ $M^{-1} \cdot s^{-1}$] of the wild-type thermolysin, respectively. The acidic and alkaline pK_as of the wild-type thermolysin were determined to be 5.3 ± 0.1 and 8.2 ± 0.1, respectively. The acidic pK_a of N112D was 5.7 ± 0.1, being 0.4 ± 0.2 units higher than that of the wild-type enzyme. The alkaline pK_a of N112D and the acidic and alkaline pK_as of N112E were almost identical with those of the wild-type thermolysin.

Shift in the pK_a Values of the Thermolysin-Catalyzed Hydrolysis of ZDFM by Mutation—ZDFM is a substrate with which the Michaelis-Menten treatment is applicable to determine k_{cat} and K_m separately. The dependence of the reaction rate on the substrate concentration was examined with respect to the wild-type enzyme, N112D, and N112E at 25°C and at pH 5.5–8.5 (Fig. 4). The reaction rates of N112D and N112E were drastically decreased compared with the wild-type thermolysin. For the wild-type enzyme at pH 5.5–7.5 and the mutants N112D and N112E at pH 5.5–6.5, a saturated profile of the Michaelis-Menten kinetics was obtained, and the k_{cat} and K_m values were separately determined. For the wild-type thermolysin at pH 8.0 and 8.5 and N112D and N112E at pH 7.0–8.5, the profile of the Michaelis-Menten kinetics was not

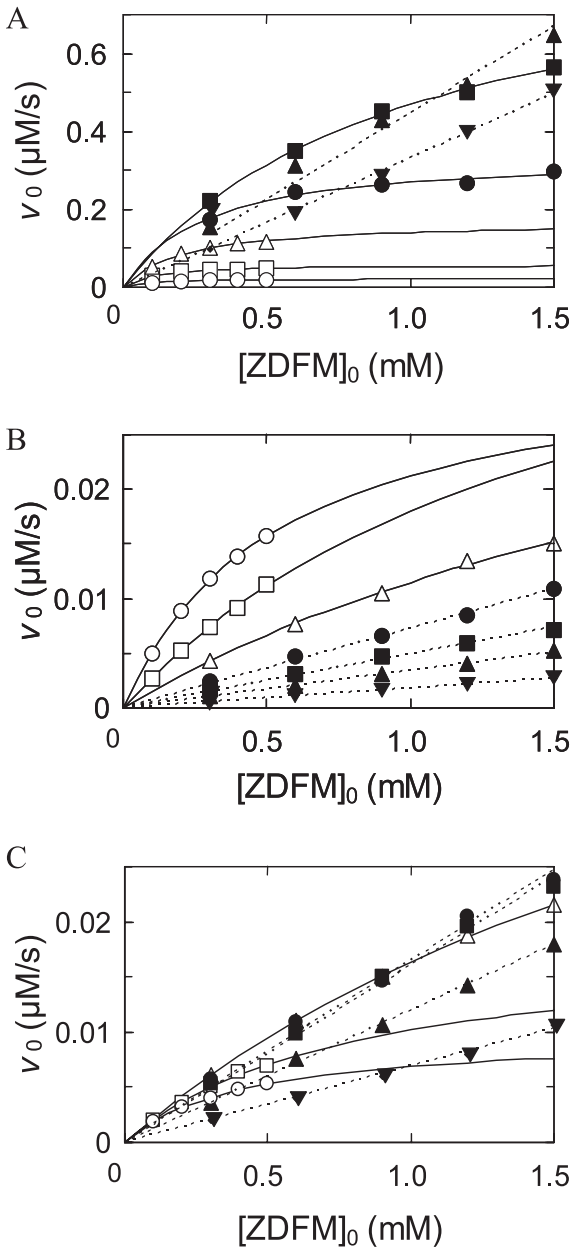

Fig. 4. **Dependence on the substrate concentration of the reaction rate of the thermolysin-catalyzed hydrolysis of ZDFM.** The reaction was carried out with the wild-type thermolysin at the concentration of 0.1 μM (A), N112D at 0.25 μM (B), and N112E at 0.25 μM (C), at 25°C and at pH 5.5 (open circles), pH 6.0 (open squares), pH 6.5 (open triangles), pH 7.0 (solid circles), pH 7.5 (solid triangles), pH 8.0 (solid squares), and pH 8.5 (inverted triangles). Solid lines represent the best fit to the experimental data using the Michaelis-Menten equation with the nonlinear least-squares methods, and broken lines represent the best fit using the linear function with the linear least-squares methods.

applicable, and k_{cat}/K_m was determined. The pH-dependences of the kinetic parameters thus obtained are shown in Fig. 5. The k_{cat}/K_m value of the wild-type thermolysin showed a bell-shaped curve with the optimum pH around 7 with pK_{e1} and pK_{e2} of 6.3 ± 0.2 and 7.8 ± 0.2, respectively (Fig. 5C). This pK_{e1} for ZDFM is considerably higher than that (5.2–5.4) for FAGLA. The intrinsic k_{cat}/K_m

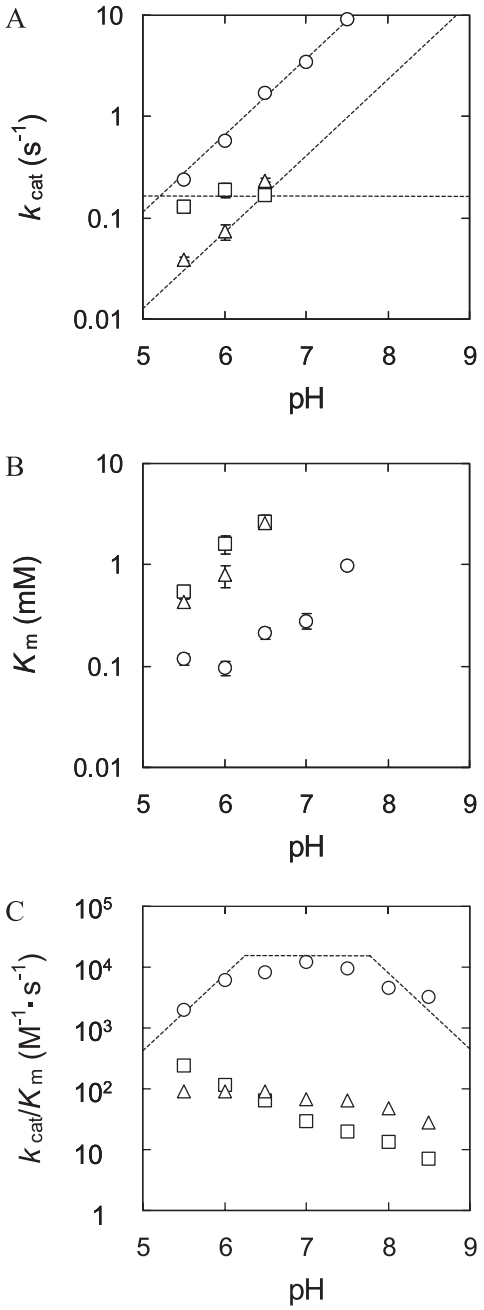

Fig. 5. **Effect of pH on the kinetic parameters of the thermolysin-catalyzed hydrolysis of ZDFM.** The pH-dependence of the morecular activity (k_{cat}) (A), Michaelis constant (K_m) (B), and the specificity constant (k_{cat}/K_m) (C) for the wild-type thermolysin (open circles), N112D (open squares), and N112E (open triangles) at 25°C. The reaction was carried out with the wild-type enzyme at the concentration of 0.1 μM and N112D and N112E at 0.25 μM at 25°C and at pH 5.5–8.5. For the wild-type thermolysin at pH 8.0 and 8.5 and N112D and N112E at pH 7.0–8.5, k_{cat} and K_m were not determined separately, and k_{cat}/K_m was obtained based on the best-fitting the experimental data to the linear function with the linear least-squares methods as shown in Fig. 4.

value was 1.9×10^4 $M^{-1}\cdot s^{-1}$, which is in good agreement with those reported previously (*11*). In the case of the mutants, the k_{cat}/K_m value decreased drastically with increasing pH from 5.5 to 8.5 (Fig. 5C). The logarithmic value of k_{cat}/K_m decreased almost linearly with increasing pH, with a slope of 0.5 for N112D and 0.2 for N112E, and no clear pK_{e2} was detected, indicating that the ionization of the catalytic residue, if any, did not affect the activity (k_{cat}/K_m) in the pH range examined. This suggests that if the ionization of the catalytic residue of the enzyme (not of the enzyme-substrate complex) does affect the activity, the pK_{e2} must be lower than 5.5. On the other hand, the logarithmic value of k_{cat} increased almost linearly with increasing pH, with a slope of 1.0 for the wild-type thermolysin and N112E, but the slope for N112D is almost zero. This suggests that the ionization of the acidic residue is observed with the wild-type thermolysin and N112E enzymes, and the pK_{es1} should be higher than 7.5 for the wild-type thermolysin and 6.5 for N112E in the enzyme-substrate complex. But, the pK_{es1} might be lower than 5.5 for N112D. The k_{cat}/K_m value of the mutants was much lower than that of the wild-type thermolysin, ranging from 1/190–1/430 at pH 7.0 to 1/8–1/22 at pH 5.5. The difference can be ascribed to the pH-dependence of k_{cat} and K_m, namely, the k_{cat} value at pH 5.5–6.5 of the wild-type thermolysin increased with increasing pH from 5.5 to 6.5, while that of N112D was almost constant in this pH range (Fig. 5A). The K_m value of the wild-type thermolysin was almost constant at pH 5.5–6.5, while those of N112D and N112E increased with increasing pH from 5.5 to 6.5 (Fig. 5B).

DISCUSSION

Cause of the Shift in pK_a Values upon Introducing a Charge at the Active Site of Thermolysin by Site-Directed Mutagenesis—Tailoring the pH-dependence of catalytic activity is one of the targets of protein engineering, especially for enzymes in industrial use. Thermolysin has been industrially used extensively for the synthesis of ZDFM, a precursor of a synthetic sweetener, aspartame (*13*).

In this study, we changed the acidic pK_a (pK_{e1}) from 5.3 to 5.7 by the single mutation Asn112 to Asp in the hydrolysis of FAGLA. We presume that a negative charge introduced to position 112 stabilizes the protonation of Glu143 or Zn^{2+}-OH, resulting in an increase in the pK_a value by 0.4 units. In the hydrolysis of ZDFM, however, no such acidic pK_a shift was demonstrated: the activity expressed by k_{cat}/K_m of the mutant enzymes did not exhibit the bell-shaped curve and was remarkably decreased with increasing pH from 5.5 to 8.5 (Fig. 5C). This is probably due to electrostatic repulsion of the negative charges between the Asp or Glu residue introduced and the Asp residue of ZDFM. Therefore, in order to shift the optimal pH to the alkaline side without loss of the activity, a mutation that stabilizes the protonation of Glu143 or Zn^{2+}-OH but has no interaction with Asp of ZDFM is required. Our results also suggest the possibility that introduction of a positive charge at the active site may shift the optimal pH to the acidic side. We examined the expression of N112K, N112H, and N112R, even though a protein band of 34.0 kDa corresponding to thermolysin was not observed on SDS-PAGE (Fig. 2).

The prepro-forms of thermolysin and its mutant enzymes are designed to be expressed in the thermolysin expression system used in the present study, and it is known that their mature forms are produced in the culture medium of the recombinant cells (*27*). The mechanism of

the conversion of the prepro-forms to mature ones is not yet known. Probably, the prepro-forms folded in the recombinant cells are activated autocatalytically by cleaving the peptide bond at the end of the prepro-sequence. One possible reason that the proteins corresponding for N112K, N112H, and N112R were not observed is that the prepro-forms of these mutant enzymes might not fold correctly due to an electrostatic effect by the introduced positive charge, and thus they might be digested by proteases of the host cells. On the other hand, it is reported that Asn112 forms a part of the S2' subsite, and the carbonyl group of the side chain interacts with a substrate by a hydrogen bond (*37*). Therefore, another possibility is that the mutant enzymes might lose autocatalytic digestion activity. The prepro-forms could not then be converted to the mature ones autocatalytically, even if they were in the correctly folded conformation, and consequently they could become substrates for proteases in the host cells.

Modification of the pH-dependence of the enzyme activity by introduction of polar residues to the active site was first reported with subtilisin BPN' (*38*, *39*) and most recently with *Bacillus licheniformis* α-amylase as well (*40*). The design of mutants, however, has not been rationalized because the microenvironment of the active site is complex, and evaluation of electrostatic interaction is difficult. Instead, the optimal pH of xylanase, chitinase, and β-amylase were successfully changed by substituting amino acids based on the sequence comparison with their family enzymes with different pK_a values (*41–43*).

*Analysis of the Shift in p*K$_a$ *Values by the Point Charge Model*—According to the point charge model in the previous papers (*18*, *44–46*), the increment in pK_a, ΔpK_a, for an ionizable group (the charge of which is Z_1e) is given by the following formula:

$$2.303\ RT\ \Delta pK_a = NZ_1Z_2e^2/(D\ r) \quad (3)$$

Using the numerical values for the electronic charge $e = 4.80 \times 10^{-10}$ esu, Avogadro's number $N = 6.02 \times 10^{23}$; the valence of the charge $Z_1 = 1$, and that of the second charge $Z_2 = 1$, the distance between the two charges r = 5.3 Å, the gas constant $R = 8.31 \times 10^7$ erg·deg^{-1} mol^{-1}, the absolute temperature T = 298 K, and the experimentally obtained $\Delta pK_a = 0.4$, the dielectric constant D is calculated to be 115. The D value of the protein surfaces so far reported is in the range of 40–55 (*39*, *45*, *46*), and the value 115 is unrealistically high, suggesting that the point-charge model expressed by Eq. 3 is not applicable to the system of thermolysin and that there might be a change in microenvironmental-charged state or structure in the active site involved in the pK_{a1}-shift induced by the site-directed mutagenesis at Asn112.

In conclusion, the pH-dependence of the activity of thermolysin was changed by changing Asn112, but the change is restricted to a neutral substrate and the degree of change is limited. An extensive study to modify the pH-dependence is currently underway by the library screening as well as amino-acid point-mutation.

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