Application Potency of Engineered G159 Mutants on P1 Substrate Pocket of Subtilisin YaB as Improved Meat Tenderizers

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A serine protease, subtilisin YaB, produced by alkalophilic *Bacillus* YaB, shows promises as a potent meat tenderizer, because its substrate specificity is for small amino acids, which are found at high levels in meat connective tissue proteins. Substrate specificity engineering of the substrate binding pockets was used to generate more suitable meat-tenderizing mutants, G124A, G124V, G159A, and G159S, derived from recombinant wild subtilisin YaB and expressed in *Bacillus subtilis* DB104. The characteristics of these recombinant enzymes were studied to evaluate their usefulness as improved meat tenderizers. The proteolytic activities of recombinant subtilisin YaB, engineered subtilisin YaBs, and commercially available papain, bromelain, collagenase, and elastase were compared using elastin, collagen, casein, and myofibrillar proteins as substrates. Hydrolysis of beef proteins was evaluated using the myofibrillar fragmentation index and collagen solubility. The results demonstrated that recombinant mutant G159A was the most improved meat tenderizer and can be used in the meat pH range of 5.5−6.0 and the temperature range of 10−50 °C. Contrary to the result obtained from artificial substrate, mutant enzymes engineered on G124 residues did not exhibit better tenderizing ability when elastin, collagen, or meat was used as substrate, suggesting the necessity of evaluation by real substrate before protein-engineered enzymes are applied commercially.

KEYWORDS: Application potency; protein-engineered subtilisin YaB; G124; G159

INTRODUCTION

Proteins are the principal functional and structural components of processed meats (*1*). Among their sensory characteristics, tenderness is the most important factor (*2*). Meat toughness is subdivided into "actomyosin toughness", attributed to changes in myofibrillar proteins, and "background toughness", attributed to connective tissue (*3*, *4*). One approach to increasing meat tenderness is to significantly reduce the amount of connective tissues without causing extensive degradation of myofibrillar proteins (*5*). This aim may be achieved by mechanical destruction or, alternatively, the use of proteolytic enzymes, such as papain, bromelain, and ficin, derived from plants. However, these enzymes, used commercially as meat tenderizers in America and Europe, often lead to overtenderization and off-flavor due to the broad substrate specificity and the bitter peptides produced (*6*). An ideal meat tenderizer would be a proteolytic enzyme specific for collagen and elastin.

Microbial proteases are another source of potent meat tenderizers (*6−8*). A microbial alkaline elastase, YaB (subtilisin YaB, EC 3.4.21.14), produced extracellularly by alkalophilic

Bacillus YaB (*9*, *10*), shows promise as a good meat tenderizer because of its markedly superior specificity for elastin and collagen compared to other commercial meat tenderizers and other microbial proteases, such as the well-studied subtilisin (*5*, *11−13*). The subtilisin YaB gene, *ale*, has been cloned and sequenced (*14*), and its translation efficiency has been optimized for optimal secretion yield (*15*). However, its high sequence homology with subtilisin resulted in its being reassigned to the subtilisin family as subtilisin YaB.

An ideal meat tenderizer would be a protease with substrate specificity for connective tissue proteins, such as collagen and elastin, both of which are rich in small amino acids, such as alanine (*16*). Subtilisin YaB shows promise as a potent meat tenderizer with high substrate specificity for alanine residues (*5*) but still acts on tyrosine and phenylalanine residues. To engineer subtilisin YaB as an improved meat tenderizer, engineered enzymes have been constructed from subtilisin YaB by replacing amino acids Gly124 and Gly159 in the S1 substrate binding pocket, G124 being located in the waist of the pocket and G159 at the bottom. To restrict the substrate binding site toward smaller amino acids, such as alanine and glycine, which are present at high levels in the meat connective tissue proteins, collagen and elastin, protein engineering of Gly124 and Gly159 into amino acids with larger side chains or more hydrophilic amino acids, such as valine, alanine or serine, will restrict the

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space and hydrophobicity within the pocket, thus restricting the substrate specificity to smaller amino acids. Recently, this enzyme was engineered by site-directed mutagenesis to restrict access to the binding pocket toward small amino acids by replacing Gly124 with an alanyl residue or valyl residue (mutants *G124A* and *G124V,* respectively) (*16*). However, recombinant subtilisin YaB retains the ability to bind and cleave substrates containing amino acids with large side chains, such as phenylalanine and tyrosine, at the S1 substrate binding pocket, thus limiting its use as a meat tenderizer. To construct a more favorable meat tenderizer, the mutants *G159A* (G159 mutated into alanine) and *G159S* (G159 mutated into serine) were also constructed. In this study, the collagen and elastin specificities of all these enzymes were tested and compared to their activities against myofibrillar protein. The applicability of these recombinant mutant enzymes as improved meat tenderizers was examined.

MATERIALS AND METHODS

Materials. Papain, bromelain, collagenase, elastase, and the substrates casein, elastin, collagen, and albumin were purchased from Sigma Chemical Co. (St. Louis, MO). A "Seprasorb" protein purification kit was purchased from Risma Biotechnology Co. (Taipei, Taiwan). Myofibrillar proteins were prepared as described by Kimura and Maruyama (*17*). Molecular weight standard proteins were purchased from Amersham (Chennal, India). Media were purchased from Difco Co. (Ann Arbor, MI). All other chemicals were purchased from Sigma or Merck (Rahway, NJ).

Bacterial Strain, Plasmids, and Site-Directed Mutagenesis. *Bacillus subtilis* DB104 (*nprE18 nprR2 DaprE3 his-101*) (*18*), kindly provided by Dr. Doi (University of California), was used as the host cell. The construction of plasmid pEX600A and of the substrate binding site-engineered *G124A* and *G124V* enzyme mutants has been previously described (*16*); mutants *G159A* and *G159S* were constructed in the same method by using synthetic oligonucleotides 5′-CAGGTGCAG-GAAATGTTGCATTCCCAGCACGCTAT-3′ and 5′-CAGGTGCAG-GAAATGTTAGTTTCCCAGCAGCGTAT-3′ to construct mutants *G159A* and *G159S* separately.

Expression and Purification of Recombinant Subtilisin YaB and Mutant Subtilisin YaB. Plasmids containing the wild-type and mutant *ale* genes were transformed into *B. subtilis* DB104 by protoplast transformation as described previously (*19*). Transformants harboring each plasmid were isolated and grown for 48 h at 37 °C in LB medium supplemented with 10 μg/mL tetracycline. The recombinant wild-type and mutant enzymes were partially purified by ultrafiltration and QM-cellulose chromatography (Seprasorb kit) as previously described (*20*) and were stored at 0−4 °C.

Assays of Enzymatic Activity. Caseinolytic activity was measured using casein as substrate as described previously (*12*). To assay enzyme activities at various temperatures or pH values, the procedures were modified by changing the temperature or the buffer; the buffers used were 0.02 M HC₃COOH−CH₃COONa (pH 3.0−6.0), 0.02 M KH₂-PO₄−Na₂HPO₄ (pH 5.0−7.0), 0.02 M Tris-HCl (pH 7.0−9.0), and 0.02 M Na₂CO₃−NaHCO₃ (pH 9.0−11.0). To determine thermostability and pH tolerance, the enzymes were preincubated for 30 min at various temperatures or pH values before assay.

Hydrolysis of Elastin, Myofibrillar Protein, and Collagen. Hydrolysis of elastin, myofibrillar protein, and collagen was assayed by a modification of the above caseinolytic activity assay. The commercial enzymes papain, bromelain, collagenase, and elastase were dissolved in H₂O and adjusted to 100 units of caseinolytic activity/mL before each assay as well as recombinant and mutated subtilisin YaBs. One hundred microliters of each enzyme was incubated for 1 h at 37 °C with shaking with 20 mg of substrate in 0.5 mL of buffer (50 mM Tris-HCl buffer, pH 6.0, 1 mM CaCl₂), and then the reaction was stopped by the addition of 0.7 mL of TCA solution (0.11 M trichloroacetic acid, 0.22 M CH₃COONa, and 0.33 M CH₃COOH). After 25 min of incubation at room temperature, the precipitate was removed

by centrifugation (12000*g*; 4 °C and 20 min), and the absorbance of the supernatant was measured at 275 nm. One unit of proteolytic activity was defined as 1 mg of tyrosine released per minute (extinction coefficient of 0.0061 for 1 μg/mL of tyrosine). The enzyme concentration was determined using a DC protein assay kit (Bio-Rad Co., Richmond, CA) with bovine serum albumin as the standard protein.

Sodium Dodecyl Sulfate—Polyacrylamide Gel Electrophoresis (SDS-PAGE). The hydrolytic effect of enzymes on myofibrillar protein was also determined by SDS-PAGE. Five milligrams of substrate and 100 units of enzyme were dissolved in 0.5 mL of 50 mM Tris-HCl buffer containing 1 mM CaCl₂, pH 6.0, and incubated for 15 h at 8 °C or for 1 h at 45 °C; then, 0.5 mL of sample buffer (24 mM Tris-HCl, pH 6.0, 10% glycerol, 0.8% SDS, 0.2 mL of mercaptoethanol, and 0.04% bromophenyl blue) was added, and the sample was boiled for 5 min to stop the reaction. The extent of hydrolysis was determined by SDS-PAGE, as described by Laemmli (*21*).

Rheometer Test of Breaking Force. Beef was cut into several 1 cm³ cubes, each of which was injected in the center and each face with enzyme solution and then immersed in 0.01% enzyme in distilled water; the total amount of enzyme applied (injection and immersion) in each cube was equivalent to 5% of the meat in weight, for 15 h at 10 °C, and then the beef cubes were incubated for 20 min at 70 °C before mechanical measurement. The mechanical texture of each beef cube was measured using a rheometer (CR-200D, Sun Scientific Co., Ltd.) with a 19 mm wide tooth-type plunger parallel to the grain of the muscle fiber, at a table speed of 20 mm/min and a force of 5 kg. The breaking force was defined as the force required to penetrate 90% of the meat sample.

Measurement of Myofibrillar Fragmentation. The hydrolysis of meat proteins by recombinant wild-type and mutant enzymes and by collagenase, elastase, and the commercially used meat tenderizing enzymes bromelain and papain was determined by indirectly measuring myofibrillar fragmentation as described previously (*22*). Beef (0.1 g) was chopped and incubated for 30 min at 37 °C in 0.05 M Tris-HCl buffer, pH 6.0, with each enzyme (adjusted to 100 units of caseinolytic activity to modify the commercial application condition); then, the mixture was centrifuged (12000*g*; 4 °C and 15 min) and myofibrillar protein extracted from the precipitate and lyophilized as described previously (*5*). The myofibrillar protein was then suspended in deionized H₂O at a final concentration of 0.25 mg/mL, and the extent of myofibrillar fragmentation was measured from the absorbance at 540 nm.

Collagen Solubilization. Collagen solubilization was determined according to the method of Reddy (*23*). The meat was enzyme-treated as described for the myofibrillar fragmentation test and centrifuged (12000*g*; 4 °C and 15 min), and a 100 μL aliquot of the supernatant was taken for hydroxyproline determination. The precipitate was dissolved in 1 mL of 0.03 M NaCl, heated at 77 °C for 1 h, and then centrifuged (12000*g*; 4 °C and 15 min); the precipitate dissolved in 100 μL of H₂O and the supernatant were taken for hydroxyproline determination. The hydroxyproline content of the two supernatants relative to the total hydroxyproline content (first supernatant plus second supernatant and second pellet) was used as a measure of collagen hydrolysis.
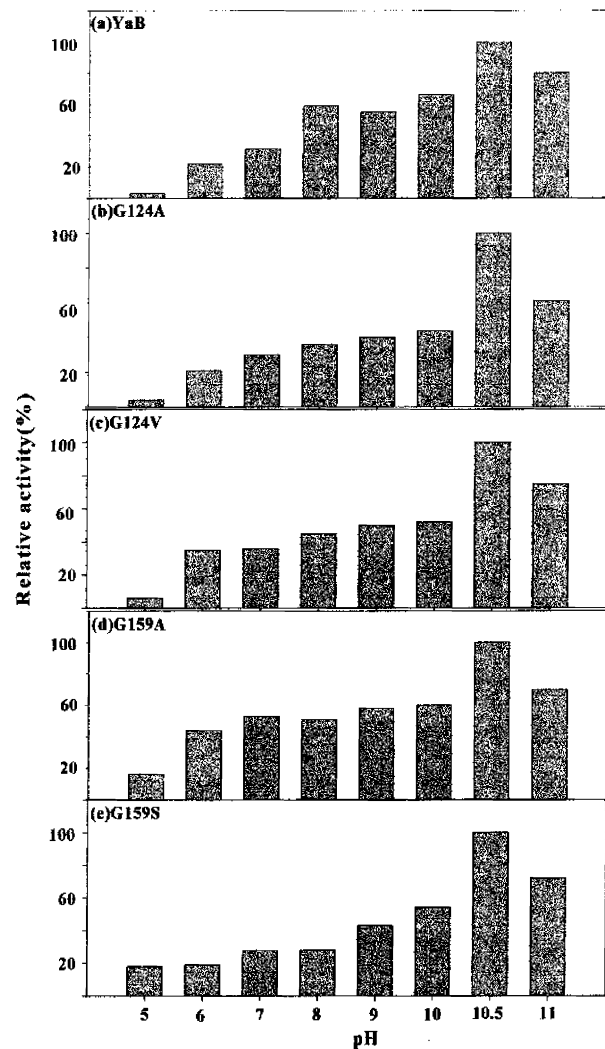
RESULTS AND DISCUSSION

Enzyme Activity of Engineered Recombinant Subtilisin YaB. To investigate the activity of recombinant subtilisin YaB and the protein-engineered mutant enzymes at various conditions of temperature and pH, caseinolytic activity was measured. As shown in **Figure 1**, all showed maximal activity at 55 °C; at 75 °C, no activity was detected, whereas at 10 °C, ∼10% of maximal activity was maintained. The results show that, although they are less active at lower temperatures, these mutant enzymes can still be used at refrigeration temperatures. Geesink et al. (*24*) tested the effect of incubation temperature (in the absence of added enzymes) on the tenderness of lamb longissimus muscle meat and found that temperatures >25 °C during the pre-rigor and early post-rigor periods adversely affected
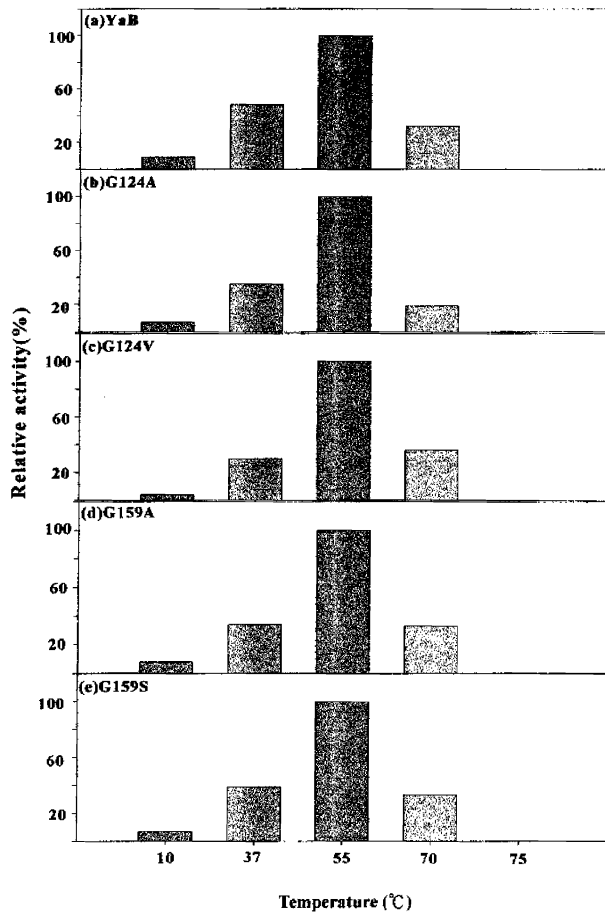
Figure 1. Activity of recombinant subtilisin YaB and engineered subtilisin YaB at various temperatures. One hundred percent relative activity represents 336, 135, 195, 117, or 116 units/mL for recombinant subtilisin YaB or mutant G124A, G124V, G159A, or G159S at pH 10.5, respectively. The experiment was performed as described in the text.

tenderness (explained by an effect on muscle shortening and/ or reduced proteolysis of myofibrillar proteins), whereas a temperature at rigor onset of ∼15 °C was optimal for tenderness (minimal muscle contraction). Application of an enzyme specific for connective tissue proteins at a low temperature for long time intervals or, alternatively, at room or higher temperatures for short time intervals can result in better meat tenderness. The temperature profiles (activity relative to the maximum at 55 °C) for the recombinant subtilisin YaB and mutant enzymes were almost identical.

The activity of these recombinant enzymes at various pH values was also tested. As shown in **Figure 2**, recombinant YaB and all mutant enzymes showed higher activity in the pH range of 8−11. The low activity at pH 5 may be due to the p*I* of casein; however, the mutant enzymes *G159A* and *G159S* retained ∼20% of their maximal activity at pH 5, although the activities of mutants *G124A* and *G124V* were lower. Wiklund et al.(*25*) examined the optimal pH for bovine meat tenderness during aging and found that myofibrillar protein solubility was linearly dependent on pH over the range from 5.5 to 6.8, whereas the myofibrillar fragmentation index was pH-independent. An ideal meat tenderizer should produce low myofibrillar protein degradation and high connective tissue protein degradation. Using an ideal meat tenderizer at a meat pH of pH 5.5−6.0 and a refrigeration temperature of ∼15 °C could reduce changes in myofibrillar proteins, and the enzyme reaction could be limited or stopped by changing the pH. However, to investigate their characteristics as an ideal meat tenderizer, it was necessary

Figure 2. Activity of recombinant subtilisin YaB and engineered subtilisin YaB at various pH values. One hundred percent relative activity represents 283, 115, 123, 112, or 100 units/mL for recombinant subtilisin YaB or mutant G124A, G124V, G159A, or G159S at pH 10.5 and 55 °C, respectively. The experiment was performed as described in the text.

to investigate the enzyme activities of recombinant subtilisin YaB and protein-engineered subtilisin YaB in terms of elastin, collagen, and myofibrillar proteins.

Hydrolysis of Elastin, Collagen, Casein, and Myofibrillar Proteins. To test the effect of these enzymes on connective tissue proteins, elastin, collagen, and myofibrillar protein were used as substrates in hydrolysis assays, and the results were compared to those obtained using the commercially used meat tenderizers papain and bromelain; collagenase and elastase were also used for comparison. The specific activity ratios were estimated as the collagen degradation/myofibrillar protein degradation (Co/M) ratio and the elastin degradation/myofibrillar protein degradation (E/M) ratio. **Table 1** shows that recombinant mutant *G159A* exhibited a better Co/M ratio than collagenase and a better E/M ratio than elastase, whereas mutant *G159S* also had a high Co/M ratio but no higher than that of collagenase. Mutants *G124A* and *G124V* were better than the wild-type enzyme in that they had slightly higher E/M ratios, but their collagen specificities were not improved (mutant *G124V* having the lowest Co/M ratios), indicating too strict a limitation of the substrate binding pocket for collagen. Papain and bromelain showed broad specificities, resulting in low E/M and Co/M ratios, whereas collagenase and elastase were specific

Table 1. Specific Elastin, Collagen, Casein, and Myofibrillar Protein Hydrolysis Ratios for Recombinant Wild-Type Subtilisin YaB, Engineered Subtilisin YaBs, and Commercially Available Enzymes

	specific hydrolysis ratio[a]	
	E/M[b]	Co/M[b]
subtilisin YaB	1.00	1.00
G124A	1.69	1.07
G124V	1.55	0.10
G159A	4.45	13.80
G159S	0.99	4.56
papain	1.89	0.42
bromelain	1.34	1.21
collagenase	0.97	10.75
elastase	2.93	0.14

[a] The specific hydrolysis ratio is the ratio for the given enzyme relative to the ratio for subtilisin YaB. [b] E/M and Co/M represent, respectively, the elastin hydrolysis/myofibrillar protein hydrolysis ratio and the collagen hydrolysis/myofibrillar protein hydrolysis ratio. Each enzyme was adjusted to 100 units of caseinolytic activity/mL before each assay.

for collagen and elastin, as expected. Surprisingly, mutation of Gly124 did not result in high E/M ratios, as Mei reported by using purified recombinant enzyme and artificial substrate elastin−orcein and myofibrillar proteins (*16*). In Mei's study, the assay was carried out at the optimal pH of 10.5 and an artificial substrate, elastin−orcein, was used, whereas, in the present study, the assay was performed under conditions more similar to those in meat (pH 6.0 and partially purified enzymes) and the result is therefore more representative of the application potency of these recombinant mutant enzymes. Takagi et al. (*26*) replaced Gly127 in subtilisin E, corresponding to Gly124 of subtilisin YaB, with Ala, Ser, and Val and found that the P1 pocket of these mutants showed a marked preference for amino acids with small side chains. Mutagenesis of Gly124 or Gly151, located in the waist of the subtilisin YaB P1 pocket, also resulted in mutants showing a marked preference for small substrates (*12*). Moreover, Gly124, reported to be an important residue with a clear influence for the P1, P2, P3, and P4 substrate residues, is conserved in all subtilisins (*27*). This result indicates a possible influence on other binding sites. The question of the influence of mutation of Gly124 in subtilisin YaB on other pocket residue changes has been raised (*16*). In this regard, argument about the substrate specificity of G124 mutants toward real substrates before the application of these mutants still needs to be evaluated. Another mutation site has also been engineered at the bottom of the P1 pocket. In this position, when a conserved Gly in subtilisin BPN′ was replaced by nonionic amino acids, the catalytic efficiency toward small hydrophobic substrates was increased (*28*). When the corresponding Gly159 of subtilisin YaB was mutated into Ala (mutant *G159A*) or Ser (mutant *G159S*), both enzymes showed better substrate specificity toward elastin and collagen, indicating that both should be improved meat tenderizers in terms of substrate specificity when elastin and collagen are tested.

Surprisingly, mutations of the Gly124 residue in subtilisin YaB did not exhibit better substrate specificities as expected, and the activities of G124 mutants were lower as well. From these results, it is clear that mutation of residues at the bottom of the P1 pocket restricts the substrate binding pocket when elastin and collagen are used as substrates, whereas mutation in the waist of the P1 pocket does not have the same result. This result also suggests that the application potency should be evaluated by using real substrate more than artificial substrates prior to application. This point of view was also highlighted by Chobert et al. (*29*). When β-casein was used as substrate,
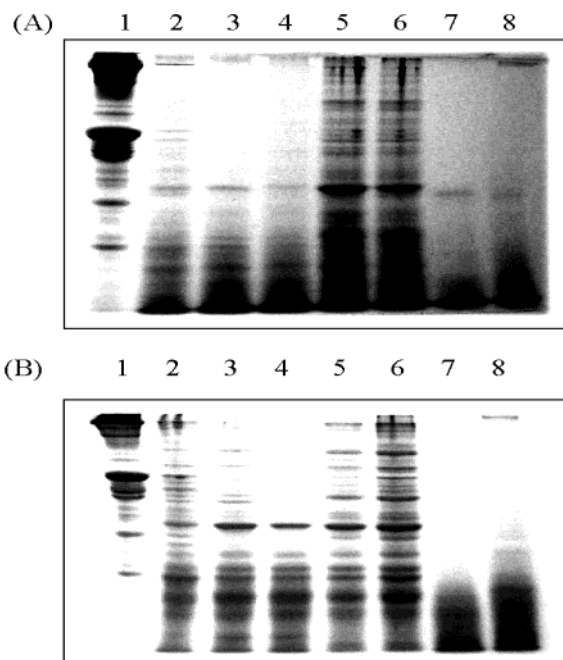
Figure 3. SDS-PAGE profile of myofibrillar protein degradation products after digestion with recombinant subtilisin YaB and engineered subtilisin YaB and commercially available enzymes: (A) after incubation for 1 h at 45 °C; (B) after incubation for 15 h at 8 °C; lanes 1, control (no enzyme); lanes 2, recombinant subtilisin YaB; lanes 3, mutant G124A; lanes 4, mutant G124V; lanes 5, mutant G159A; lanes 6, mutant G159S; lanes 7, papain; lanes 8, bromelain. The experiment was performed as described in the text.

unexpected cleavage on amidated amino acids other than cleavage on artificial substrates puzzled the trypsin's substrate engineering on K188. Why would the G159 mutants exhibit better substrate specificity toward real substrate than G124 mutants more restricted on artificial substrate? Although the G124 mutants exhibited more restricted substrate specificity toward the artificial substrate carbobenzoxyalanine-*O-p*-nitrophenyl ester and carbobenzoxyglycine-*O-p*-nitrophenyl ester, other cleavage sites still presented when insulin A or B chain was used as substrate (this will be discussed in another paper). From our results, although substrate binding toward artificial substrate in G124 within the P1 pocket was strictly restricted, it may limit the substrate binding affinity toward real substrate. On the other hand, G159 mutants only narrow the bottom space by introducing extra methyl (*G159A*) or methanol (*G159S*) groups, both pointing inside the pocket, and no interaction with artificial substrate was predicted by modeling; however, G159 mutants exhibit higher activities toward elastin and collagen. The substitution of Gly159 into a more hydrophobic residue, alanine, shows better activity toward hydrophobic substrates (elastin and collagen) than substitution by an uncharged polar residue, serine.

We also examined myofibrillar protein hydrolysis by SDS-PAGE. As shown in **Figure 3**, after 1 h of incubation at 45 °C or 15 h of incubation at 8 °C, mutants *G159A* and *G159S* were less active than the wild-type or other mutants on myofibrillar proteins. This result, together with the hydrolysis ratio study, indicates the potential of mutants *G159A* and *G159S* as improved meat tenderizers.

Myofibrillar Fragmentation Index and Collagen Solubilization. From previous data, it is necessary to evaluate the application potency by using meat. Tenderization of beef treated with engineered enzymes was checked by measuring the

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Table 2. Fragmentation Index and Collagen Solubility of Meat Treated with Wild-Type and Engineered Recombinant Subtilisin YaB[a]

treatment	% fragmentation index[b]	% soluble collagen[b]
control[c]	100	7
wild-type	127	22
G124A	117	33
G124V	114	34
G159A	109	36
G159S	119	41
bromelain	159	26
collagenase	137	77
elastase	154	23
papain	164	55

[a] Each enzyme was adjusted to 100 units of caseinolytic activity/mL before application to meat. [b] % fragmentation index and % soluble collagen represent the hydrolysis of meat myofibrillar protein and meat collagen after each given enzyme treatment, repectively. [c] Control data were obtained from nontreated meat.
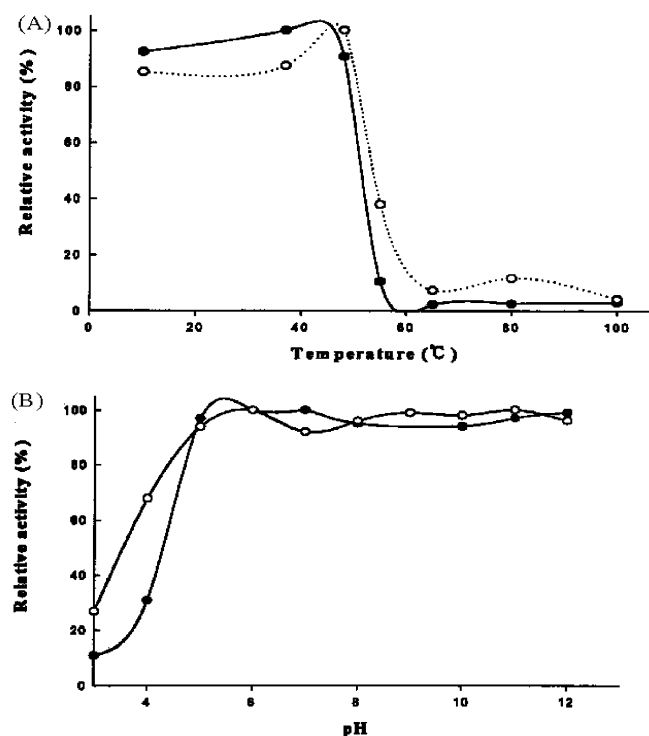
Figure 4. Thermal stability (A) and pH stability (B) of recombinant subtilisin YaB (●) and G159A mutant subtilisin YaB (○). One hundred percent relative activity represents 284 and 102 units/mL for recombinant YaB and the G159A mutant in the thermal stability study and 162 and 86 units/mL for recombinant YaB and the G159A mutant in the pH stability study.

physical breakage force. The force required to penetrate beef cubes was reduced when recombinant subtilisin YaB, mutant enzymes, papain, or bromelain was applied. However, the breaking force using mutants *G159A* and *G159S* was no lower than that using papain or bromelain (data not shown). Because the ideal meat tenderizer would be an enzyme that specifically hydrolyzes collagen and not myofibrillar protein, the breaking force is not a good indication of ideal tenderization. The myofibril fragmentation index and collagen solubilization in enzyme-treated beef were therefore examined. As shown in **Table 2**, mutant *G159A* had the lowest fragmentation index, representing the least myofibrillar degradation. Collagenase and papain produced more soluble collagen, but hydrolysis of myofibrillar proteins (fragmentation index) was not negligible. The recombinant enzymes resulted in more collagen hydrolysis

than bromelain or elastase. Again, we confirmed the application potency of mutants *G159A* and *G159S* as improved meat tenderizers, with mutant *G159A* appearing to be the best of these mutant enzymes, whereas G124 mutants exhibited lower activities toward collagen and elastin when beef was used as substrate. The results also indicate the necessity of evaluation by real substrate.

pH Stability and Thermal Stability of Potent Meat Tenderizers. The pH stability and thermal stability of the *G159A* mutant were evaluated. As shown in **Figure 4**, the *G159A* mutant was stable between pH 4 and 12, including the pH range seen with meat (pH 5.5−6). When the thermal stability was examined, mutant *G159A* had the same profile as the wild-type enzyme; after 15 min of incubation, both lost ∼50% of their activity at 50−55 °C and were completely inactivated at temperatures >60 °C. The result suggests the application convenience of the recombinant mutant enzyme *G159A*, because it can be used at refrigeration temperatures; the reaction can be stopped by changing the pH or simply increasing the temperature to >60 °C.

In conclusion, it is necessary to evalute the application potency by using real substrate more than artificial substrate for engineered enzymes prior to application. G159 mutants are better meat tenderizers than G124 mutants, contrary to the evaluation obtained in the case of artificial substrates. Among the mutants tested, *G159A* appeared to be the most improved meat tenderizer and can be used at refrigeration temperatures; the activities can be stopped by changing the pH or simply increasing the temperature to >60 °C.

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