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Analysis of Autodegradation Sites of Thermolysin and Enhancement of Its Thermostability by Modifying Leu[155] at an Autodegradation Site

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The relationship between the autodegradation and thermostability of thermolysin (TLN) was studied. Four autodegradation sites in TLN were identified in the presence of Ca^{2+}. One of the sites was identified as Gly[154]-Leu[155], and Leu[155] was substituted with various amino acids, X = Ala, Ser, Phe, and Gly, by site-directed mutagenesis. The thermostability at 80°C increased with the amino acid substitutions in the order of Ala>Phe>Ser>Gly>Leu (WT TLN). An additional autodegradation fragment that was not observed with WT TLN appeared for all mutant TLNs examined. The autodegradation site shifted from the Gly[154]-Leu[155] bond to the X[155]-Ile[156] one with the mutation at Leu[155]. Furthermore, the Ile[164]-Asp[165] bond was recognized newly as an autodegradation site in the mutant TLNs for the production of AF3′.

Key words: thermolysin, autodegradation, thermostability, protease.

Thermolysin (TLN, EC3.4.24.27), which is produced by *Bacillus thermoproteolyticus* and *B. stearothermophilus*, is well known as a thermostable protease in the presence of calcium ions (*1–3*). Since TLN shows higher stability in an organic solvent (*4–6*) and/or in the presence of a high concentration of salt (*7–9*), the enzyme is widely used for the industrial synthesis of an artificial sweetener, aspartame (*4*, *5*). Its stability and proteolytic mechanisms have been extensively investigated (*9–15*).

The TLN gene has been cloned, sequenced, and expressed in *B. subtilis* (*2*). Its amino acid sequence (*16*) and three-dimensional structure (*17–19*) have been determined. We have constructed several activated or stable mutant enzymes by means of site-directed mutagenesis to obtain higher levels of TLN gene expression, to change the proteolytic activity, and to enhance the stability (*12*, *15*). Several attempts to stabilize proteases have been carried out by introducing disulfide bonds and hydrophobic interactions (*20–22*).

Proteases are inactivated at elevated temperatures through denaturation (unfolding) of their protein structures and through autodegradation. Autodegradation might be affected by the substrate specificity of a protease. The autodegradation sites of *B. subtilis* neutral protease are known to be peptide bonds with Met, Leu, Ile, and Ala at the P1′ position according to the nomenclature of Schechter and Berger (*23*), and the autodegradation sites correspond well to the substrate specificity (*24*). Substitution of amino acids at the autodegradation sites could make proteases more resistant to autodegradation.

On the other hand, autodegradation of proteases is also influenced by the location of the susceptible bonds in the protein structure. When subtilisin was mutated by creating Ca^{2+} binding sites, the mutant enzyme became more stable, and autodegradation was suppressed (*25*).

We reported previously that heat inactivation of TLN at 80°C was caused mainly through autodegradation (*14*). In this paper, we describe the autodegradation sites in TLN, and demonstrate that autodegradation is strongly suppressed in mutant enzymes in which an amino acid residue (Leu[155]) located at one of the autodegradation sites is replaced with other amino acids. The effect of such a mutation on the protein structure is also shown.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—*Escherichia coli* K12 JM109 [*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, *Δ* (*lac-proAB*), *F′* (*traD36*, *proAB*$^+$ *lac*^{Iq}, *lacZΔM15*)] and *E. coli* BMH71$^-$13 mutS [*hi*, *supE*, *Δ* (*lac-proAB*), *mutS::Tn10*, F′ (*proAB*$^+$, *lac*^{Iq}, *lacZΔM15*)] were used for site-directed mutagenesis (*26*). The *Bacillus* strain used was *B. subtilis* MT-2 (*trpC2*, *leuC7*, *hsrM*, *hsmM*, Npr$^-$) (*2*). The plasmids used were pMK4 (Ap^R, *nprM*) (*27*) and pTB51 (Km^R) (*28*). Bacteria were grown in Luria-Bertani medium (LB medium, 1% peptone, 0.5% yeast extract, 0.5% NaCl). LC agar (LB medium plus 1% casein and 1.5% agar) was used for the detection of proteases. The antibiotics used were kanamycin (5 μg ml^{-1}) and ampicillin (50 μg ml^{-1}).

Autodegradation of TLN—A three-times crystallized preparation of TLN (Lot CKK 7034) was purchased from Wako Pure Chemicals, Osaka, Japan. TLN (30 μM) was dissolved in 50 mM Tris-HCl buffer, pH 7.0, containing 5 mM $CaCl_2$ or 5 mM EDTA, and then incubated at a specified temperature for 30 min. The solution was analyzed by SDS-polyacrylamide (20% acrylamide) gel electrophoresis (SDS-PAGE). SDS-PAGE was performed according to the procedure of Lammli (*29*). Proteins were

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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 used was a product of Pharmacia (Uppsala, Sweden). The marker proteins were rabbit muscle phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), chicken ovalbumin (43 kDa), bovine erythrocyte carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and bovine milk α-lactalbumin (14 kDa).

Determination of the N-Terminal Amino Acid Sequences of the Fragments Formed on Autodegradation—The fragments formed on autodegradation were isolated by SDS-PAGE, and then electroblotted onto a PVDF membrane (Bio-Rad, Hercules, CA, USA) using a semidry-blotting system (Biocraft Co., Tokyo, Japan). After staining with Coomassie Brilliant Blue, the fragments were isolated from the membrane and then their N-terminal amino acid sequences were analyzed with a PPSQ-10 automated protein sequencer (Shimadzu, Kyoto, Japan).

Site-Directed Mutagenesis—A GeneEditor™ *in vitro* site-directed mutagenesis system (Promega, Madison, WI, USA) was used for site-directed mutagenesis. The following mutagenic oligonucleotides were used: 5′TTGATAAATGGCGCCGGCTGTAT3′ (L155A); 5′TGATAAATGAATCCGGCTGTA3′ (L155F); 5′TTGATAAATGGATCCGGCTGTA3′ (L155S); and 5′TTGATAAATGCCTCCGGCTCTA3′ (L155G). Mismatches are underlined. Four kinds of mutation were introduced into the TLN gene, and the proteolytic activity of the mutant enzymes was detected on a LC agar plate. The mutations were confirmed by DNA sequencing. A DNA sequencer (LI-COR, Lincoln, NE, USA) and a Thermo Sequenase cycle sequencing kit (Amersham Life Science, Cleveland, OH, USA) were used for DNA sequencing, and the primer (5′ ATCCGTTCATTATAGCCAAGGC 3′) for the DNA sequencing was chemically synthesized.

Purification of the Enzyme—The enzyme secreted into the culture supernatant was salted-out with ammonium sulfate (60% saturation), and the precipitate was dissolved in 50 mM Tris-HCl buffer, pH 7.0, containing 1M ammonium sulfate and 5 mM $CaCl_2$. The enzyme solution was loaded onto a Phenyl-Toyopearl 650M column [size: 1.0 cm (inner diameter) × 10.0 cm, Tosoh, Tokyo, Japan], which had been equilibrated with the same buffer. The fractions containing protease activity were collected, and then subjected to gel-filtration HPLC on a TSKgel G3000SW column [size: 7.8 mm (inner diameter) × 30 cm, Tosoh] with 50 mM Tris-HCl buffer, pH 7.0, containing 5 mM $CaCl_2$. The purified enzyme was confirmed by the single band on SDS-PAGE.

Enzyme Properties—A three-times crystallized and lyophilized preparation of TLN (Lot T5CB491; 8720 proteinase units/mg according to the supplier) was purchased from Daiwa Kasei, Osaka, Japan. This preparation was used as an authentic TLN preparation without further purification. The solution of TLN was filtered through a Millipore membrane filter, Type HA (pore size: 0.45 μm) before use. Protease activity was assayed with azocasein (Sigma, St. Louis, MO, USA; Lot 101K7023) as a substrate (*30*). The enzyme solution (30 μM) in 50 mM Tris-HCl buffer, pH 7.0, containing 5 mM $CaCl_2$ was incubated at 80°C for 0–30 min, and then the solution was kept at room temperature for 5 min, followed by determination of the remaining activity.

The kinetic parameters, Michaelis constant (K_m) and catalytic constant (k_{cat}), were determined from Lineweaver-Burk plots. The WT and mutant TLNs were determined by means of hydrolysis of a synthetic peptide substrate *N*-carbobenzoxy-glycyl-L-leucine amide (Z-Gly-Leu-NH_2) (Peptide Institute Inc., Osaka, Japan) at concentrations of 3, 5, 7.5, 10, and 12.5 mM. The reaction was carried out at 37°C in 50 mM Tris-HCl buffer, pH 7.0, containing 5 mM $CaCl_2$. At 10 min intervals, aliquots (200 μl) were removed from the reaction mixture, and 100 μl of acetic acid was added to stop hydrolysis. H_2O (700 μl) and 1 ml of a ninhydrin solution (ninhydrin, 1 g; hydrindantin, 150 mg; 2-methoxy ethanol, 37.5 ml; and 5 M sodium acetate buffer, pH 5.5, 12.5 ml) were added to the solution, followed by boiling for 15 min (*31*). After that, 5ml of 50% (v/v) ethanol was added, and the protein concentration was monitored as the absorbance at 570 nm. L-Leucine amide (Leu-NH_2) was used as a standard, and the data were fitted to the Michaelis-Menten equation.

Circular Dichroism (CD)—A Jasco J-720 (Tokyo, Japan) spectropolarimeter equipped with a Peltier system for cell temperature control was used for CD measurements. The system was routinely calibrated with an aqueous solution of recrystallized *d*-10 camphorsulphonic acid. Ellipticity is expressed as mean residue molar ellipticity [θ] (deg cm^2 $dmol^{-1}$). The spectrometer conditions were typically: spectral range 195–260 nm, 100 mdeg sensitivity; 0.1 nm resolution; 4 s response time; 20 nm min^{-1} scan rate; and 7 accumulations. A control baseline was obtained with a solvent and all the components without the proteins. CD spectra were recorded at 25°C using a 1-mm cell for the far-ultraviolet wavelength range. The protein concentration of the samples was 10 μM in 5 mM Tris-HCl buffer (pH 7.0). CD spectra were processed with Jasco software, and finally expressed in mean-residue molar ellipticity units.

RESULTS

Analysis of the Autodegradation Sites in TLN—As the autodegradation of TLN at 80°C occurred very rapidly, the fragments were difficult to detect on SDS-PAGE. Accordingly, further autodegradation experiments were carried out at 70°C. TLN (30 μM) was incubated in the presence of 5 mM $CaCl_2$ or 5 mM EDTA at 70°C for 30 min, and then the autodegradation of TLN was examined. TLN was rapidly autodegraded, three main fragments of 26, 25, and 14 kDa appearing in the presence of 5 mM EDTA (Fig. 1). This result was in good agreement with that already reported (*32*, *33*). Autodegradation was also examined in the presence of 5 mM $CaCl_2$, with which TLN is known to be more stable than in the presence of EDTA. The autodegradation progressed slowly under these conditions, and a greater amount of undegraded TLN (34.6 kDa) remained in comparison with in the presence of EDTA. At least ten fragments were observed on autodegradation of TLN in the presence of 5 mM $CaCl_2$, and this autodegradation pattern was clearly different from that in the presence of 5 mM EDTA (Fig. 1).

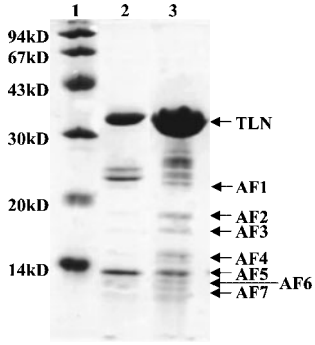

Fig. 1. **Autodegradation of TLN.** Lane 1: molecular mass marker proteins; lane 2: TLN autodegradated by incubation in the presence of 5 mM EDTA; lane 3: TLN autodegradated by incubation in the presence of 5 mM $CaCl_2$. The N-terminal amino acid sequences of AF1 to AF7 were determined. The autodegradation of TLN was examined by incubating 30 μM TLN in 50 mM Tris-HCl buffer, pH 7.0, in the presence of 5 mM $CaCl_2$ or 5mM EDTA at 70°C for 30 min.

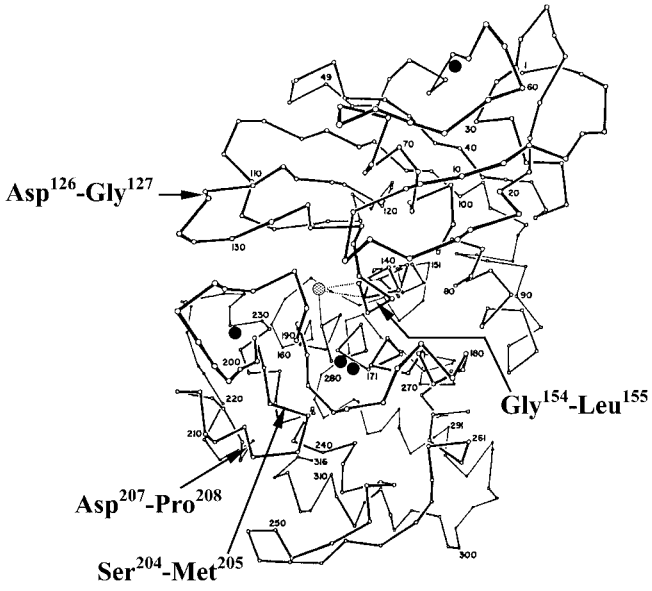

Fig. 2. **The autodegradation sites of TLN in the three-dimensional (3-D) structure of TLN.** The autodegradation sites are indicated by arrows. The 3-D structure of TLN is drawn based on Holmes and Matthews (*18*).

The fragments obtained on autodegradation of TLN in the presence of $CaCl_2$ were analyzed to identify the autodegradation sites. Table 1 shows the N-terminal amino acid sequences of seven fragments (AF1 to AF7). Based on the results, the autodegradation sites were judged to be Asp^{126}-Gly^{127}, Gly^{154}-Leu^{155}, Ser^{204}-Met^{205}, and Asp^{207}-Pro^{208} (Fig. 2). One site, Ser^{204}-Met^{205}, was previously found in the presence of EDTA (*32*, *33*). However, the other sites were newly identified in the presence of 5 mM $CaCl_2$. In this study, we focused on the autodegradation of TLN without EDTA but with 5 mM $CaCl_2$.

Site-Directed Mutagenesis of TLN at Leu155—If we could construct a mutant TLN that is tolerant to autodegradation by means of site-directed mutagenesis, the mutant TLN is expected to be more stable than WT TLN. Amino acid substitutions were made considering the substrate specificity of TLN, so that cleavage at the autodegradation site by TLN could be suppressed. Gly^{127}, Met^{205}, and Pro^{208}, being the N-terminal amino acids of the AF-1, AF-5, AF-6, and AF-7 fragments, respectively, are located in the highly conserved regions in a family of *Bacillus* neutral proteases (*16*, *34*). This suggests that these residues might be significant as to the structures and functions of these proteases. Accordingly, it could be considered that TLN activity might decrease on mutation of these residues when they are replaced by other amino acids. On the other hand, Leu^{155}, which corresponds to the N-terminal amino acid of the AF-3 fragment, is uniquely located in the helical region connecting the N-terminal and C-terminal domains of TLN (*17*). This residue is not conserved in all *Bacillus* neutral proteases, and thus mutation of this residue could have less effect on the enzyme activity. On the basis of these considerations, Leu^{155} was selected as the residue for amino acid substitution in the present study.

Leu^{155} was replaced by Ala, Phe, Ser, and Gly, and the mutant enzymes thus constructed were designated as L155A, L155F, L155S, and L155G, respectively. The mutant plasmid pMK4 was sub-cloned into pTB51 to construct a *Bacillus* expression vector, and the resultant plasmid was named pBKN1-4 (Fig. 3).

Characterization of the mutant TLNs—The protein secretion levels of the mutant enzymes, as evaluated by SDS-PAGE, were almost equal to that of WT TLN (data not shown). The hydrolysis of Z-Gly-Leu-NH_2 catalyzed by the WT and mutant TLNs was measured at 37°C and pH 7.0. The enzyme concentrations were adjusted to 30 μM. It was essentially impossible to estimate kinetic parameters K_m and k_{cat} separately from the Lineweaver-Burk plots because of the low solubility (~12.5 mM) of the substrate (Z-Gly-Leu-NH_2) and of the fairly high K_m values (10–20 mM). Thus, they were roughly estimated

Table 1. **N-terminal amino acid sequence of each autodegradation fragment.**

Autodegradation fragment	Molecular mass (kDa)	N-terminal amino acid sequence	Autodegradation site*
AF1	22	Gly-Gln-Thr-Phe-Ile	Asp^{126}-Gly^{127}
AF2	18	Ile-The-Gly-Thr-Ser	Ile^{1}
AF3	17	Leu-Ile-Tyr-Gln-Asn	Gly^{154}-Leu^{155}
AF4	15	Ile-Thr-Gly-Thr-Ser	Ile^{1}
AF5	12	Met-Ser-Asp-Pro-Ala	Ser^{204}-Met^{205}
AF6	11	Pro-Ala-Lys-Tyr-Gly	Asp^{207}-Pro^{208}
AF7	10	Pro-Ala-Lys-Tyr-Gly	Asp^{207}-Pro^{208}

*The numbers next to amino acids are the positions in mature TLN. AF2 and AF4 are both N-terminal fragments of TLN starting from Ile^{1}.

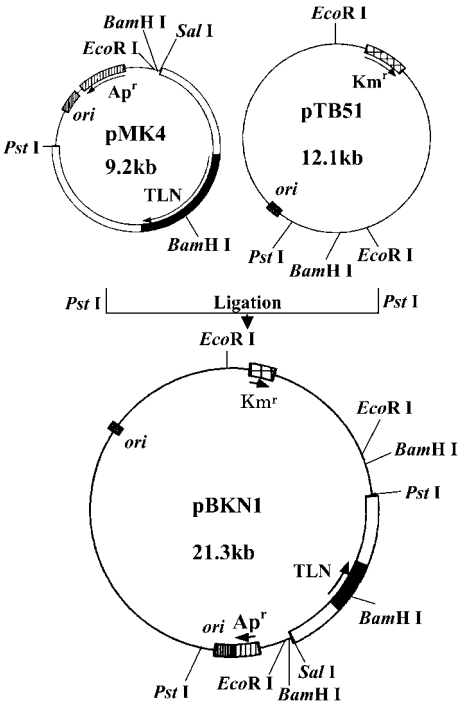

Fig. 3. **Construction of the TLN expression plasmid.** The closed bar indicates the TLN gene. The position and direction of transcription of the TLN gene are indicated by arrows. Plasmids pTB51 and pMK4 were digested with *Pst* I, and each fragment was ligated with T4 DNA ligase. The resultant plasmid was named pBKN1.

(Table 2). It was found that the mutation of Leu[155] mainly influenced k_{cat}, and that the effect of the mutation on K_m was much less or almost negligible. The specificity constants, k_{cat}/K_m, of L155S and L155G were significantly increased to 350–400% compared with those (220 min^{-1} mM^{-1}) of WT TLN and L155A, which was similar to that of WT TLN. The k_{cat}/K_m value of L155F, however, decreased to 40% to that of WT TLN. The effects of the amino acid substitutions at position 155 on the kinetic parameters were in the order of Ser > Gly > Ala > Leu

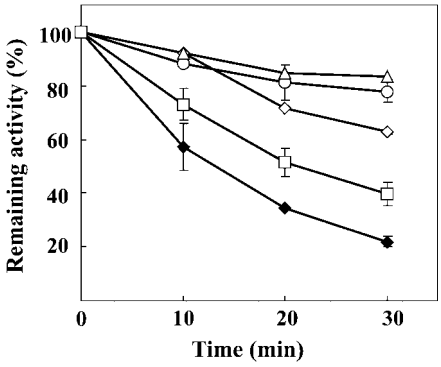

Fig. 4. **Thermostability of the WT and mutant TLNs.** The purified enzyme solutions (30 μM) were kept in 50 mM Tris-HCl buffer, pH 7.0, containing 5 mM $CaCl_2$ at 80°C for the indicated times, and then cooled quickly, and the remaining activities were measured at 37°C with azocasein as the substrate. Solid diamonds, WT TLN; open diamonds, L155A; open circles, L155F; open diamonds, L155S; open squares, L155G.

Table 2. **Kinetic parameters of the WT and mutant TLNs.**

TLN	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
WT TLN	14	3,100	220
L155S	13	9,900	760
L155G	10	9,000	900
L155A	20	4,800	240
L155F	17	1,400	80

Assays were carried out using Z-Gly-Leu-NH_2 as the substrate at 37°C and pH 7.0.

(WT TLN) > Phe for k_{cat}, and Gly > Ser > Ala > Leu (WT TLN) > Phe for k_{cat}/K_m. The remarkable activation observed with substitution of Leu155 by Ser and Gly could be considered due to the stabilization of the transition state rather than that of the ES complex. The kinetic parameters for WT TLN were comparable to those previously reported (*7*). With *N*-furylacryloyl-glycyl-L-leucine amide (FA-Gly-Leu-NH_2 or FAGLA), K_m, k_{cat}, and k_{cat}/K_m were > 3mM > 5,400 min^{-1}, and 1,800 mM^{-1} min^{-1}, respectively, at pH 7.4 and 25°C. On changing of the Z-group in Z-Gly-L-Leu-NH_2 to an FA group, the K_m values were considerably reduced suggesting the formation of a more stable ES complex, but the k_{cat} values for the two substrates were rather similar. It is considered that the activation energy required to convert the ES complex to the transition state is essentially the same for the two substrates.

CD spectra of the WT and mutant TLNs were obtained to determine the secondary structure. The spectra of the WT and mutant TLNs in the far UV region were the same as those previously reported for WT TLN, suggesting that the protein structures of the mutant TLNs were fully folded, the structures being the same as that of WT TLN (*35*, data not shown). The K_m values of the mutant TLNs were almost the same as that of WT TLN, and they showed comparable enzyme activities, as shown by the k_{cat} and k_{cat}/K_m values. This also suggests that the mutant TLNs have fully folded structures. The kinetic parameters of the WT and mutant TLNs well reflected the similarities of their CD spectra.

Thermostability of the mutant TLNs—When the WT (30 μM) and mutant TLNs (30 μM) were incubated at 70°C for 30 min, the remaining activity was almost 100%, *i.e.* essentially no loss of activity was observed. Therefore, the temperature of 80°C was selected for TLN incubation to examine the thermostability of TLN. The WT and mutant TLNs were incubated at 80°C for different periods of time (0–30 min), followed by incubation at 37°C for 30 min, and then the remaining activities were measured. The remaining activities of all the mutant TLNs were higher than that of WT TLN (Fig. 4). When WT TLN was incubated at 80°C for 30 min, the remaining activity was only 20%, whereas those of L155G, L155S, L155F, and L155A were 40, 65, 80, and 85%, respectively. The thermostability depends on the amino acid residue at position 155 in the order of Ala > Phe > Ser > Gly > Leu (WT TLN). From the substrate specificity of TLN, it can be expected that TLN cleaves Gly-Ala and Gly-Phe bonds more favorably than Gly-Ser and Gly-Gly ones, and thus L155S and L155G were predicted to be less susceptible to autodegradation, and more stable as to thermal treatment than L155A and L155F. However, L155A and

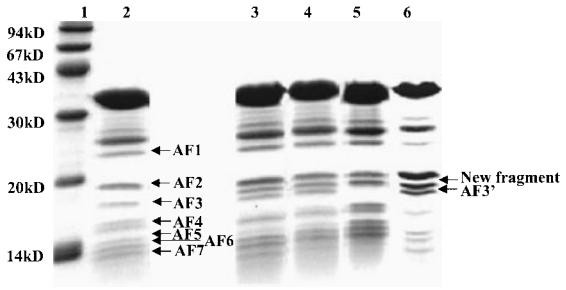

Fig. 5. **Autodegradation of the mutant TLNs.** Lane 1: molecular mass marker proteins; lane 2: WT TLN; lane 3: L155S; lane 4: L155G; lane 5: L155F; lane 6: L155A. The autodegradation of TLN (30 μM) was examined in 50 mM Tris-HCl buffer, pH 7.0, in the presence of 5 mM $CaCl_2$ at 70°C for 30 min.

L155F showed higher thermostability. Substituting Leu^{155} with hydrophobic amino acids such as Ala and Phe may increase the hydrophobic packaging of the tertiary structure of TLN, and this may suppress the autodegradation.

Mutational Effects on Autodegradation—To analyze the changes in the autodegradation patterns of the mutant TLNs, autodegradation was analyzed by SDS-PAGE. Fragments formed on autodegradation of the mutant TLNs were analyzed by SDS-PAGE (Fig. 5). A new fragment that was not observed for WT TLN was observed between the AF2 and AF3 fragments for all mutant TLNs. The N-terminal amino acid sequence of this fragment was determined to be Ile-Tyr-Gln-Asn-Glu, which corresponds to the sequence of Ile^{156}-Tyr^{157}-Gln^{158}-Asn^{159}-Glu^{160}. This result strongly suggests that the autodegradation at Gly^{154}-Leu^{155} was suppressed in the mutant TLNs, and that the autodegradation site of Gly^{154}-Leu^{155} observed with WT TLN had shifted to a new site, X^{155}-Ile^{156}, where X is Ala, Phe, Ser, or Gly. Next, the N-terminal sequences of AF3 fragments produced on the autodegradation of WT and all the mutant TLNs were analyzed (Table 3). The sequence for WT TLN indicates that the AF3 fragment was produced through cleavage of Gly^{154}-Leu^{155}. The sequences for mutant TLNs, L155S, L155G, and L155A, but not L155F, indicate that the AF3 fragments were produced through cleavage of the peptide bond Ile^{164}-Asp^{165}. This suggests that the AF3 fragment produced from WT TLN and those from the mutant TLNs (L155S, L155G, and L155A) must be different, although their mobility on SDS-PAGE is almost the same. Consequently, the fragments from the mutant TLNs, which appeared at the electrophoretic position of the AF3 fragment produced from WT TLN, were designated as AF3′ fragments. This suggests that the Gly^{154}-X^{155} bond is not

Table 3. **N-terminal sequences of AF3 and AF3′ fragments of the WT and mutant TLNs.**

TLN	N-terminal amino acid sequence	Autodegradation site*
WT TLN	Leu-Ile-Tyr-Gln-Asn	Gly^{154}-Leu^{155}
L155S	Asn-Glu-Ala-Ile-Ser	Ile^{164}-Asp^{165}
L155G	Asn-Glu-Ala-Ile-Ser	Ile^{164}-Asp^{165}
L155A	Asn-Glu-Ala-Ile-Ser	Ile^{164}-Asp^{165}

*The numbers next to the amino acids are the positions in mature TLN.

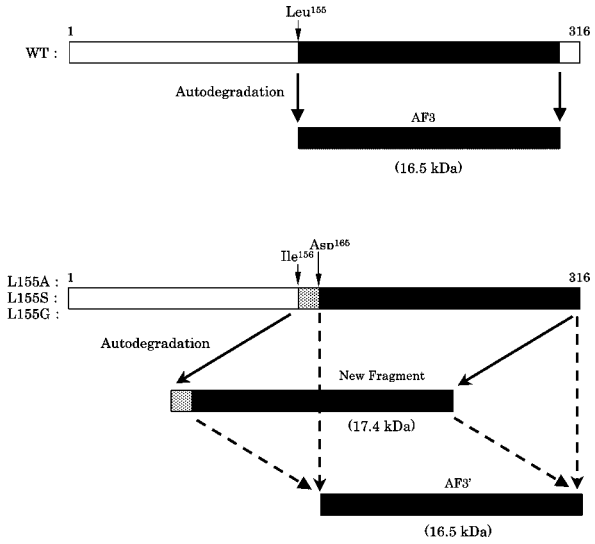

Fig. 6. **Schematic diagram of the autodegradation of TLN and L155A.**

cleaved in the mutant TLNs. The suppression of autodegradation at Gly^{154}-Leu^{155} in WT TLN on mutation of the Leu residue to another one is one of the reasons for the enhancement of the thermostability of the mutant TLNs.

Based on the autodegradation patterns of the mutant TLNs (L155S L155G, and L155A), an autodegradation model is proposed (Fig. 6). Considering the molecular size of AF3 (16.5 kDa), there should be a second cleavage site for WT TLN around position 306 (Val^{306}). On the other hand, the AF3′ fragments might be produced without secondary cleavage, and the C-terminal end of AF3′ must be that (Lys^{316}) of TLN. By replacing Leu^{155} by X (=Ser, Gly, and Ala) at the Gly^{154}-Leu^{155} bond in WT TLN, the cleavage at the Gly-X bond was avoided, but cleavage at X^{155}-Ile^{156} and Ile^{164}-Asp^{165} was induced. This suggests that a new autodegradation site, Ile^{164}-Asp^{165}, in the mutant TLNs other than L155F does not agree with the TLN substrate specificity. The Ile^{164}-Asp^{165} bond might be more exposed on the surface of the molecule with the mutation at position 155, so this position easily undergoes autodegradation. Conformational features rather than sequence characteristics might preferentially determine the site of proteolytic attack, although the autodegradation site comprising X^{155}-Ile^{156} agreed reasonably with the TLN substrate specificity. If an amino acid residue resistant to autodegradation is introduced to the most susceptible site, such as X^{155}-Ile^{156}, in the mutant TLNs, mutant TLNs with higher stability than L155S, L155G, and L155A might be possible. It should be noted that cleavage of the Ile^{164}-Asp^{165} bond did not occur in L155F, while the Phe^{155}-Ile^{156} bond but not the Gly^{154}-Phe^{155} one was cleaved.

DISCUSSION

Several attempts to suppress autodegradation by modifying the autodegradation sites have already been carried out for TLN-like proteases, although stabilization of the mutant enzymes was unsuccessful (*20*). TLNs are well known to be stabilized in the presence of $CaCl_2$ and inac-

tivated by autodegradation at the optimum temperature (80°C) of TLNs (*1*, *8*, *14*). Thermostable TLNs have been desired for industrial applications such as for the enzymatic synthesis of an aspartame precursor. If we could construct a mutant TLN resistant to autodegradation, it could exhibit improved thermostability. Four autodegradation sites in TLN were assigned in the presence of $CaCl_2$. One of them, Ser^{204}-Met^{205}, was previously reported for autodegradation in the presence of EDTA (*32*). The other three sites, Asp^{126}-Gly^{127}, Gly^{154}-Leu^{155}, and Asp^{207}-Pro^{208}, were newly identified as autodegradation sites in the presence of $CaCl_2$. Considering the substrate specificity of TLNs, they preferentially recognize a hydrophobic residue in the P1′ site, and an amino acid with a smaller side chain is accommodated in the P1 site (*9*, *19*). The autodegradation sites identified in this study correspond well with the substrate specificity of TLN (*17*). Moreover, all autodegradation sites were found in flexible regions on the surface of TLN.

In this study, Leu^{155} was selected as a target for site-directed mutagenesis, in order to enhance the stability of TLN, and was substituted with various amino acids (Ser, Phe, Ala, and Gly). The thermostability of the mutant TLNs was considerably increased. It was shown that autodegradation at Gly^{154}-Leu^{155} was suppressed in the mutant TLNs. Considering the substrate specificity, L155G and L155S are expected to be more resistant to autodegradation than L155A, L155F, and WT TLN. However, the thermostability increased in the order of Ala>Phe>Ser>Gly>Leu, suggesting that the enhancement of thermostability is not always due to the substrate specificity at the autodegradation site. Because the k_{cat}/K_m values of these mutant TLNs for a synthetic substrate were almost the same as that of WT TLN, it is assumed that the conformation of the active site of TLNs might be well maintained even in the mutant TLNs. Probably, the local conformations around Gly^{154}-X^{155}-Ile^{156} and Ile^{164}-Asp^{165} change significantly, and the conformational changes seem to cause the shift of the autodegradation site. The autodegradation pattern also changed, and an additional fragment of 17.5 kDa was observed. However, autodegradation might be suppressed totally in the mutant TLNs, and subsequently the thermostability of the mutant TLNs seems to be enhanced. In the future, if new autodegradation sites are designed to be more resistant to autodegradation, more thermostable mutant TLNs would be created.

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