The propeptide of subtilisin BPN' as a temporary inhibitor and effect of an amino acid replacement on its inhibitory activity

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Abstract The propeptide of subtilisin-family proteases is known to exhibit inhibitory activity toward a cognate protease in addition to its function as an intramolecular chaperone. For detailed investigation of its inhibitory properties, the propeptide of subtilisin BPN' was produced in *Escherichia coli*. Inhibitory activity measurements and electrophoresis showed that the propeptide was a temporary inhibitor, which was initially potent but was gradually degraded by subtilisin BPN' through specific intermediates. The main cleavage site was identified as Glu^{53}–Lys^{54}, with minor sites at Thr^{17}–Met^{18} and Met^{21}–Ser^{22}, which were located in turn regions of the propeptide in the complex with subtilisin BPN'. Since the isolated propeptide has been shown not to form a tertiary structure, these results indicate that main digestions proceed through proteolytic attack of subtilisin toward the accessible sites of the propeptide in the complex with subtilisin. Therefore, replacement of Glu53 at the main cleavage site by Asp, which is a less favorable amino acid than Glu for subtilisin, makes the propeptide a more resistant temporary inhibitor.

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Key words: Amino acid replacement; Propeptide, protease inhibitor; Subtilisin; Temporary inhibition

1. Introduction

Subtilisin BPN' is an alkaline protease secreted by *Bacillus amyloliquefaciens*, and produced from a precursor protein which possesses a prosequence composed of 77 residues between a presequence functioning as a signal peptide and a mature region of subtilisin. It has been demonstrated that the prosequence (or propeptide) is an intramolecular chaperone required for proper folding of subtilisin in vivo and in vitro [1], based on findings that the subtilisin gene which lacks the prosequence produces a mature but inactive form of subtilisin in vivo [2] and that the refolding efficiency of denatured subtilisin is markedly increased by covalent attachment of the propeptide to mature subtilisin [3,4] or exogenous addition of the propeptide to the solution [5,6]. In addition to its function as a chaperone, the propeptide of subtilisin has been shown to exhibit inhibitory activity toward subtilisin with an inhibitor constant of about 10^{-9} M [5,7–9]. Although it is initially potent, the propeptide is degraded by subtilisin [9], and thus has been termed a temporary inhibitor. Furthermore, a relationship between the chaperone ability of the propeptide and its inhibitory activity has also been demonstrated [8]. However, details of the degradation process of the propeptide by subtilisin, such as identification of the cleavage sites, are not well understood.

We have been carrying out structure–function relationship studies of protease inhibitors to clarify the mechanisms by which they inhibit proteases using site-specific mutants of *Streptomyces* subtilisin inhibitor (SSI) [10–13], ovomucoid domain 3 [14] and *Cucurbita maxima* trypsin inhibitor I (CMTI-I) [15] and natural mutants (SIL proteins) [16,17] of SSI. The results have suggested that conformational rigidity and high stability are required for permanent (not temporary) inhibition. In addition, we have shown that almost complete renaturation of denatured subtilisin BPN' can be accomplished using organic salts and mutated SSI, which had been converted to a digestible temporary inhibitor by removal of the disulfide bridge near the reactive site [18].

Based on these findings, we have considered that by successive amino acid replacements, it might be possible to make mutant proteins from the propeptide which exhibit permanent inhibition of subtilisin. Such studies would clarify not only the molecular mechanisms responsible for temporary inhibition of the propeptide, but also those responsible for inhibition of protease inhibitors toward proteases. In this study, as a first step toward achieving such functional alteration of the propeptide to a resistant form, we identified the cleavage site of the propeptide by subtilisin BPN' by sequence analysis of degradation intermediates, and examined the effects of amino acid replacements at the identified main cleavage site on the inhibitory properties of the propeptide.

2. Materials and methods

2.1. Materials

Restriction enzymes and DNA-modifying enzymes were purchased from Takara Shuzo, Toyobo and Boehringer Mannheim (Germany). Subtilisin BPN' was obtained from Sigma (St. Louis, MO, USA). Other chemicals were of reagent grade for biochemical research.

2.2. Construction of the propeptide expression system

Since we had already cloned the subtilisin gene which encodes a precursor form from *B. amyloliquefaciens* [19], the DNA fragment encoding the propeptide portion of subtilisin BPN' was amplified by the polymerase chain reaction. Primers with sequences of 5'-AAAC-CATGGCAGGGAAATCAAACGGGGAAAAGAAAT-3' and 5'-AAAGGATCCTTAGTACGCATGTGCTACGTGATCTTCTTC-3' were designed to introduce the *Nco*I site and an initiation codon, and a stop codon and the *Bam*HI site, respectively. An amplified fragment was digested with *Nco*I and *Bam*HI, and inserted into the *Nco*I–*Bam*HI site of plasmid pTV119N to produce pTVPRO. After confirmation of the nucleotide sequence by dideoxy sequencing, the cloned propeptide gene was inserted into the *Nco*I–*Bam*HI site of pET11d, which possesses a promoter of T7 RNA polymerase [20].

2.3. Expression and purification of the propeptide

Escherichia coli BL21(DE3) was transformed by the constructed expression plasmid. Large-scale culture in LB medium containing 50 μg/ml ampicillin was started by inoculation of a small-scale overnight culture. When A_{600} reached 1, expression of the propeptide gene was induced by addition of isopropyl-β-D-galactopyranoside to give a final

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concentration of 0.4 mM, followed by culture for 2 h. Cells suspended in 10 mM Tris-HCl/0.1 mM EDTA (pH 8.0) were disrupted by ultra-sonication. The cell lysate collected by centrifugation at 10 000 rpm was diluted with a 50 mM sodium acetate buffer (pH 5.5), and applied to a CM-52 column (2×15 cm). After washing the column, the propeptide was eluted with a linear gradient of 0–1 M NaCl. To improve the purity, the eluted propeptide was subjected to reverse-phase HPLC on a C18 column (L-column ODS, 4×150 mm) with an acetonitrile gradient.

2.4. Determination of protein concentration

Protein concentrations were determined spectrophotometrically using an $A^{1\%}_{280}$ of 11.7 for subtilisin BPN′ [21] and a molar absorption coefficient constant (ε_{280}) of 5120 [4] for the propeptide.

2.5. Inhibitory activity of the propeptide toward subtilisin BPN′

Subtilisin BPN′ at 364 nM and various molar ratios of propeptide were incubated at 25°C in 0.1 M Tris-HCl (pH 8.0). At 0, 10, 30, 50 min, an aliquot of 100 μl containing 1μg of subtilisin was withdrawn and added to 900 μl of 0.111 mM succinyl-L-Ala-L-Ala-L-Pro-L-Phe p-nitroanilide in the buffer, followed by monitoring of the absorbance at 410 nm. The inhibitory activity was defined as $[1-(V_i/V_o)]\times 100$ (%), where V_i and V_o are the activity of subtilisin in the presence and absence of the propeptide, respectively. For the 0 min incubation, V_i was obtained after the equilibrium of subtilisin–propeptide complex formation had been attained.

2.6. Electrophoretic analysis of the propeptide–subtilisin mixture

Subtilisin BPN′ at 364 nM and a 25-fold molar excess of the propeptide (9.1 μM) were incubated at 25°C in 0.1 M Tris-HCl (pH 8.0). After 0.5, 30, 60, 90 and 120 min of incubation, an aliquot (50 μl) of the mixture containing 0.5 μg of subtilisin was withdrawn. The proteins were precipitated with trichloroacetic acid at a final concentration of 20%, followed by washing with acetone, and then subjected to SDS-polyacrylamide gel electrophoresis.

2.7. Isolation of degradation intermediates of the propeptide and sequence analysis

Subtilisin (64 μg, 2.33 nmol) and propeptide (784 μg, 92.7 nmol) were incubated at 25°C for 20 min in 1.6 ml of 0.1 M Tris-HCl (pH 8.0). The proteins were then precipitated and subjected to reverse-phase HPLC on a C18 column, as described above. Isolated degradation intermediates were subjected to an Applied Biosystems Protein Sequencer model 476A (Foster City, CA, USA) to determine their amino-terminal sequences. The carboxy-terminal sequences were analyzed using a Hewlett-Packard model G1009A C-terminal protein sequencer (Palo Alto, CA, USA), which utilizes diphenyl phosphoroisothianatidate as a coupling reagent [22]. The major degradation intermediate (3 nmol) was digested with endoproteinase Asp-N using a substrate/enzyme ratio of 100 at 37°C overnight in 0.5 M urea/ 50 mM sodium phosphate (pH 8.0). The digested fragments were separated using reverse-phase HPLC on a C18 column, as described above.

2.8. Site-directed mutagenesis

E. coli CJ236 was transformed with a plasmid pTVPRO, and single-stranded DNA containing uracil bases was obtained by infection with the helper phage M13KO7. Replacement of the codon for Glu53 from GAA to GAT was carried out using a primer with a sequence of 5′-ACATTAAACGAT*AAAGCTGTAAA-3′, where the asterisk indicates the mismatched base, according to the method of Kunkel [23]. The mutation was verified by dideoxy sequencing, and the mutated gene was inserted into pET11d, as described for the wild type.

3. Results

3.1. Expression and characterization of the propeptide of subtilisin BPN′

Our expression system for the propeptide was essentially the same as that used by other groups, i.e. a pET system which utilized the promoter of T7 RNA polymerase inducible by IPTG [20]. The propeptide was expressed in the soluble fraction of E. coli, and purified by chromatography after disrup-

tion of E. coli by sonication. The yield was about 1.8 mg from 1 liter of E. coli culture. Amino-terminal sequence analysis showed that the expressed propeptide lacked the amino-terminal Met residue. Measurement of the circular dichroism spectra indicated that the propeptide did not have any typical secondary structures (data not shown), as described by other groups [4,7,24].

Then, the inhibitory activities of the propeptide toward subtilisin BPN′ at various ratios were measured using a synthetic substrate. As shown in Fig. 1(A), the propeptide was
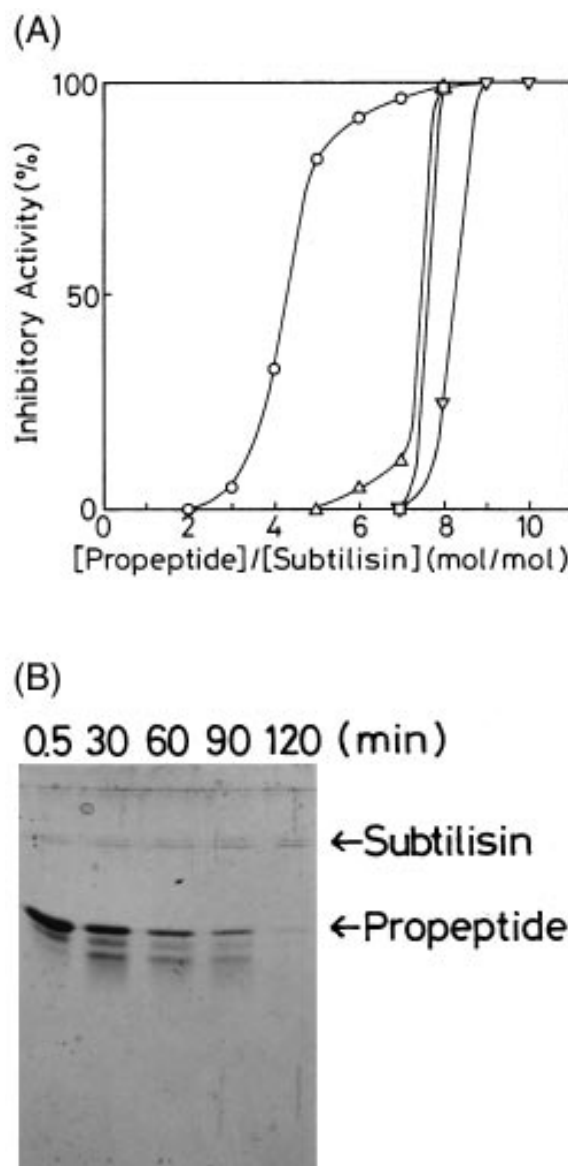

Fig. 1. Inhibitory activity (A) and electrophoretic pattern (B) of the wild-type propeptide in the reaction mixture with subtilisin BPN′. (A) Subtilisin BPN′ at 364 nM and various molar ratios of the propeptide were incubated at 25°C in 0.1 M Tris-HCl (pH 8.0). At 0 min (○), 10 min (△), 30 min (□) and 50 min (▽) of incubation, a 100-μl aliquot was withdrawn and the inhibitory activity was measured using a synthetic substrate. (B) Subtilisin BPN′ at 364 nM and a 25-fold molar excess of the propeptide were incubated at 25°C in 0.1 M Tris-HCl (pH 8.0). At the indicated time, the proteins in a 50-μl aliquot were precipitated with trichloroacetic acid and subjected to SDS-polyacrylamide gel electrophoresis (gel concentration 18.8%).
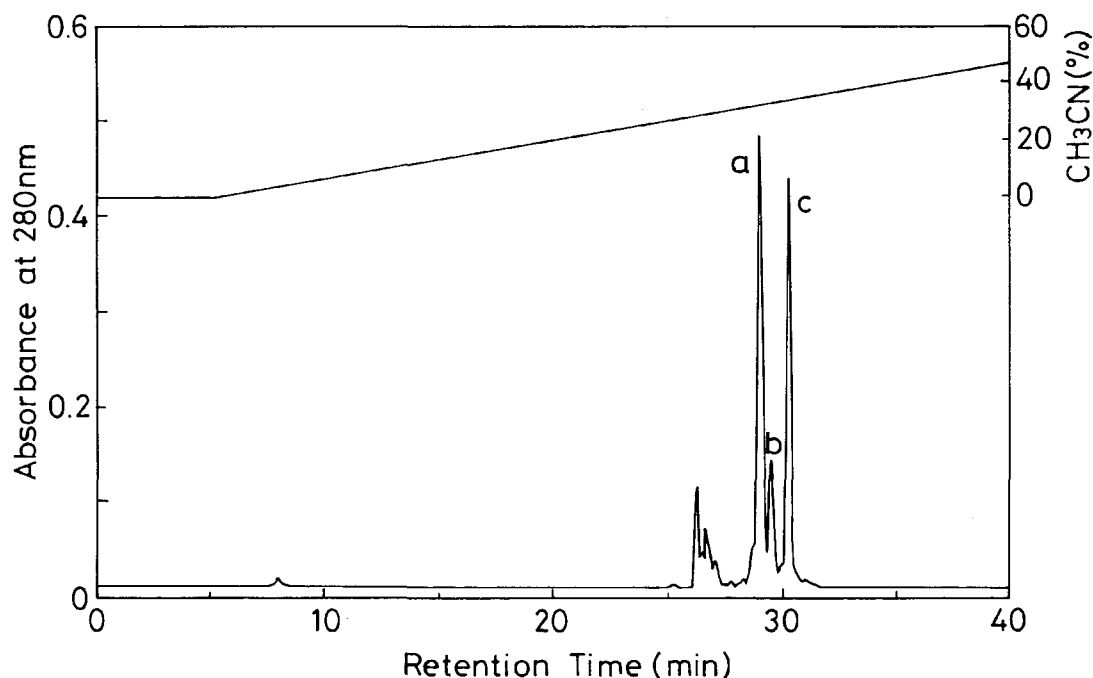
Fig. 2. Chromatogram of the subtilisin-digested propeptide on reverse-phase HPLC. Subtilisin (64 µg) and a 40-fold molar excess of the propeptide were incubated at 25°C for 20 min in 1.6 ml of 0.1 M Tris-HCl (pH 8.0). The proteins were precipitated with trichloroacetic acid, and subjected to reverse-phase HPLC on a C18 column (L-column ODS) using an acetonitrile gradient.

initially a potent inhibitor. However, as the incubation time from mixing the propeptide and subtilisin to addition of the substrate was increased, the inhibitory activities showed an evident decrease. Even at a molar ratio of 7, the inhibitory activity of the propeptide was completely lost after 30 min of incubation. This phenomenon is typical of that for a temporary inhibitor.

Since this time-dependent decrease of inhibitory activity was considered to result from degradation of the propeptide by protease, as demonstrated for a mutated SSI which was converted to a temporary inhibitor, the molecular state of the propeptide in the reaction mixture with subtilisin BPN' was investigated by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 1(B), the band of the propeptide became thinner as the incubation time increased. Concurrently, two bands which were considered to be degradation intermediates appeared below the authentic band of the propeptide; one was a rapidly migrating major band and the other was a minor band located between the propeptide and the rapidly migrating major band. These results clearly showed that the propeptide is a temporary inhibitor which is initially potent, but degraded by subtilisin through specific intermediates.

3.2. Identification of the cleavage sites in the propeptide

In order to clarify the process of propeptide degradation by subtilisin, degradation intermediates were isolated using reverse-phase HPLC. Fig. 2 shows a chromatogram of the protein sample prepared from a 20 min incubated mixture composed of subtilisin and 40-fold molar excess of the propeptide. Electrophoretic analysis indicated that peaks *a* and *b* corresponded to the major and minor intermediates, respectively, and peak *c* to the authentic propeptide. The relatively low

amount of peak *c*, compared with the result of Fig. 1(B), seems to be differences in the conditions (molar ratio and concentration) of the subtilisin–propeptide mixture. The isolated peptides were subjected to sequence analysis to identify the cleavage sites.

The amino-terminal sequence of the major intermediate was the same as that of the authentic propeptide, while Glu was identified as the carboxy-terminal residue of the intermediate. Since the propeptide possesses 6 Glu residues, the carboxy-terminal sequence was investigated by sequence analysis of its enzymatic digests. Digestion of the major intermediate by endoproteinase Asp-N produced 3 peaks on reverse-phase HPLC. Sequence analysis indicated that peaks 1, 2 and 3 corresponded to Asp^{45}-Glu^{53}, Asp^{28}-Val^{44} and Ala^1-Lys^{27}, respectively. These results clearly indicated that the major degradation intermediate corresponded to Ala^1-Glu^{53} of the propeptide, and thus the major specific cleavage of the propeptide by subtilisin occurred at Glu^{53}-Lys^{54}.

On the other hand, the carboxy-terminal sequence of the minor intermediate (-His–Val–Ala–His–Ala–Tyr) was the same as that of the authentic propeptide, while the main amino-terminal sequences were Met–Ser–Thr–Met–Ser–Ala–Ala– and Ser–Ala–Ala–Lys–Lys–Lys–Asp–, corresponding to the 18th–24th and 22nd–28th residues of the propeptide, respectively. Thus, the minor specific cleavage occurred at Thr^{17}-Met^{18} and Met^{21}-Ser^{22}. In addition, the sequences starting from the 15th and 25th residues of the propeptide were detected as minor sequences of the minor intermediates, indicating cleavages at Phe^{14}-Ser^{15} and Ala^{24}-Lys^{25}. No other peptide fragments were detected, indicating that subsequent degradation of the propeptide after the specific cleavages proceeded randomly.
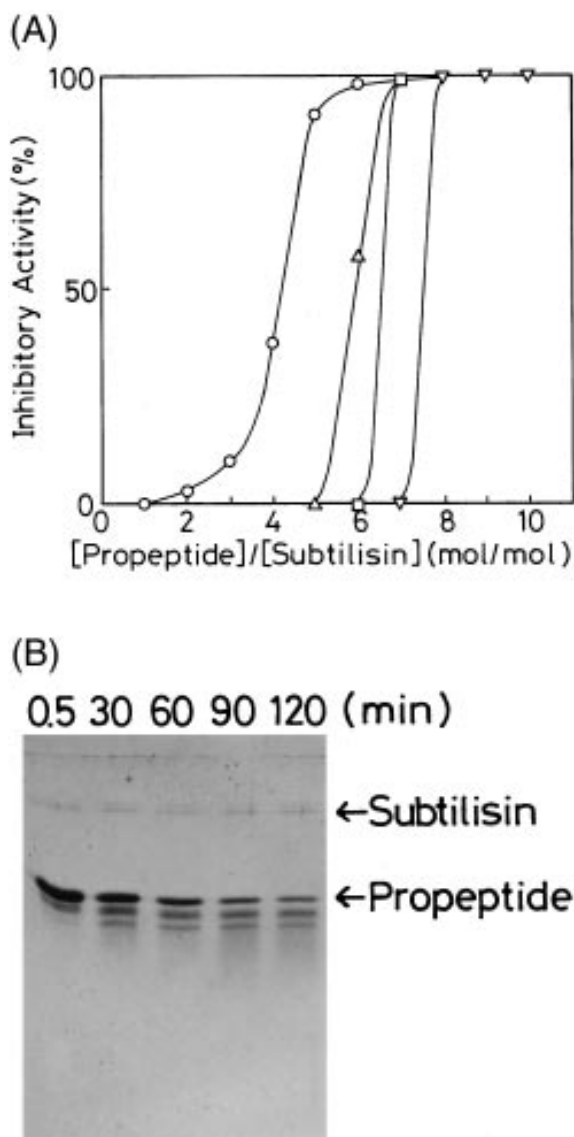
(A)

(B)

Fig. 3. Inhibitory activity (A) and electrophoretic pattern (B) of the mutated propeptide in the reaction mixture with subtilisin BPN'. The conditions were the same as those described for the wild type in Fig. 1.

3.3. Effects of an amino acid replacement on the properties of the propeptide

In order to make the propeptide more resistant to proteolytic degradation, Glu^{53} identified as the major cleavage site in the degradation intermediates was replaced. Although Glu is an unfavorable amino acid substrate of subtilisin BPN' [25], Asp or Pro has been considered to be more resistant to digestion by subtilisin BPN' from studies on the interaction of P1-site mutants of SSI and subtilisin BPN' [11]. Since Pro has an irregular main-chain configuration, Asp was introduced as the 53rd residue of the propeptide.

Fig. 3 shows the inhibitory activities and molecular states of the mutated propeptide in the reaction mixture with subtilisin BPN' at various incubation times after mixing. The mutated propeptide was degraded by subtilisin BPN' more slowly than the wild type, and consequently the rate of decrease in inhibitory activity of the mutated propeptide was slower than that of the wild type. Thus, it was shown that the replacement

$Glu^{53} \rightarrow Asp$ at the main cleavage site of the propeptide made the propeptide more resistant to proteolytic digestion by subtilisin. The CD spectrum of the mutated propeptide was the same as that of the wild type (data not shown).

4. Discussion

The propeptide of subtilisin-family proteases is a unique protein because it exhibits inhibitory activity toward a cognate protease in addition to its function as an intramolecular chaperone. Although it has also been demonstrated that the propeptide is a temporary inhibitor which is initially potent but gradually degraded by protease, subsequent detailed analyses of the propeptide as a protease inhibitor have not been carried out. In this study, we therefore attempted to clarify the process of degradation of the propeptide and then to convert it to a more resistant inhibitor.

Electrophoretic analysis of the subtilisin–propeptide mixture clearly indicated that the propeptide was degraded by subtilisin through specific intermediates which appeared as a main and minor bands, and that consequently the inhibitory activity of the propeptide toward subtilisin decreased as the incubation time was prolonged. We then identified the cleavage sites of the propeptide by sequence analysis of the degradation intermediates purified by HPLC.

The main cleavage site was identified as Glu^{53}–Lys^{54}, which was unexpected because Glu is an unfavorable amino acid substrate of subtilisin BPN' [25]. We then considered the reason for the cleavage at position 53 from the viewpoint of the three-dimensional structure. It has been demonstrated that, although the isolated propeptide hardly exhibits typical secondary and tertiary structures, it forms a defined tertiary structure by complex formation with inactive subtilisin in which there is $Ser^{221} \rightarrow Ala$ or Cys replacement at the active site [4,7,24]. The tertiary structure of the propeptide in the complex has been clarified by X-ray crystallography [26,27]. According to the revealed structure of the propeptide, Glu^{53} is located just at the turn region between the β_3-strand and α_2-helix, and thus is considered to be accessible to the proteolytic attack by subtilisin. If Glu^{53} is not at this location, a specific intermediate may not be detected, because many amino acids that are favorable as substrates of subtilisin BPN' exist around Glu^{53}. In other words, cleavage at Glu^{53} is considered to occur toward the propeptide molecule that is complexed with subtilisin by another subtilisin molecule in an uncomplexed state.

The identified minor cleavage sites Thr^{17}–Met^{18} and Met^{21}–Ser^{22} are also located just in the turn region between the β_1-strand and α_1-helix of the propeptide complexed with subtilisin BPN'. Since Met and Thr are more favorable amino acids than Glu [25], it is considered that cleavage at Thr^{17}–Met^{18} and Met^{21}–Ser^{22} proceeds more rapidly, and thereby the degradation product appears as a minor band. In contrast, minor cleavage sites in the minor intermediates were located in the secondary structures in the interior of the propeptide molecule. This situation, and the fact that these amino acids are favorable for subtilisin BPN', indicate that the cleavage at these sites may proceed when the uncomplexed propeptide is in a random-coil state. However, we could not explain why other intermediates produced by cleavage at more favorable sites were not detected. On the basis of these considerations, two pathways in the degradation of the propeptide by sub-

tilisin BPN' can be proposed: main cleavage at very accessible sites in the complex with subtilisin, and minor cleavage at the favorable residues in the random-coil state.

We then substituted Asp for Glu^{53} at the main cleavage site in order to make the propeptide more resistant to proteolytic digestion. As expected, the time-dependent inhibitory activity and electrophoretic pattern showed that the mutated propeptide became more resistant to digestion by subtilisin BPN'. Since the CD spectra indicated that hardly any structural change resulted from the replacement, conversion of the propeptide to a more resistant inhibitor was due to the resistance of the Asp residue at position 53 to proteolytic attack by subtilisin BPN'. Thus, the $Glu^{53} \rightarrow Asp$ replacement in this study was demonstrated effective for converting the propeptide to a more resistant inhibitor. However, studies using other approaches will be required for subsequent conversion, and these are now in progress.

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