Functional Analysis of the Cucumisin Propeptide as a Potent Inhibitor of Its Mature Enzyme*

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Cucumisin is a subtilisin-like serine protease (subtilase) that is found in the juice of melon fruits (Cucumis melo L.). It is synthesized as a preproenzyme consisting of a signal peptide, NH₂-terminal propeptide, and 67-kDa protease domain. We investigated the role of this propeptide (88 residues) in the cucumisin precursor. Complementary DNAs encoding the propeptides of cucumisin, two other plant subtilases (Arabidopsis ARA12 and rice RSP1), and bacterial subtilisin E were expressed in Escherichia coli independently of their mature enzymes. The cucumisin propeptide strongly inhibited cucumisin in a competitive manner with a Kᵢ value of 6.2 ± 0.55 nM. Interestingly, cucumisin was also strongly inhibited by ARA12 and RSP1 propeptides but not by the subtilisin E propeptide. In contrast, the propeptides of cucumisin, ARA12, and RSP1 did not inhibit subtilisin. Deletion analysis clearly showed that two hydrophobic regions, Asn³²–Met³⁸ and Gly⁹⁷–Leu¹⁰³, in the cucumisin propeptide were important for its inhibitory activity. Site-directed mutagenesis also confirmed the role of a Val¹³⁶-centered hydrophobic cluster within the Asn³²–Met³⁸ region in cucumisin inhibition. Circular dichroism spectroscopy revealed that the cucumisin propeptide had a secondary structure without a cognate propeptide domain and that the thermal unfolding of the propeptide at 90 °C was only partial and reversible. A tripeptide, Ile³⁵–Val³⁶–Tyr³⁷, in the Asn³²–Met³⁸ region was found to contribute toward the formation of a proper secondary structure necessary for cucumisin inhibition. This is the first report on the function and structural information of the propeptide of a plant serine protease.

Proteases play key roles in diverse processes regulating plant growth, development, and responses to environmental stimuli. They are necessary for protein turnover, strict protein quality control, and degrading specific sets of proteins. Comparative genomics analyses could provide valuable insights into the abundance and roles of various plant protease families. For example, the Arabidopsis thaliana genome has over 550 protease sequences corresponding to almost 3% of the proteome, representing all five catalytic types: serine, cysteine, aspartic acid, metallo, and threonine (1, 2). Of these, serine proteases appear to be the largest class of plant proteases, although protease activity has been demonstrated only by a few of them.

Cucumisin (EC 3.4.21.25) is an extracellular thermostable alkaline serine protease that is expressed at high levels in melon fruits (Cucumis melo L.). It comprises more than 10% of the total juice protein and is synthesized in the central parts of the fruits (3). Cucumisin is synthesized and accumulated only in melon fruits, and a cis-regulatory enhancer element in the cucumisin promoter regulates fruit-specific expression of the cucumisin gene (4). We have determined the complete nucleotide sequence of a cucumisin cDNA, the first sequenced plant serine protease, and found that cucumisin is a member of the subtilisin (EC 3.4.21.62) superfamily characterized by a catalytic triad of three amino acids: Asp, His, and Ser (5). The primary structure of cucumisin deduced from the cDNA sequence revealed that it is synthesized as a precursor consisting of four functional domains: a possible signal peptide (22 amino acid residues); NH₂-terminal prosesequence (88 residues); 54-kDa protease domain (505 residues); and 14-kDa COOH-terminal polypeptide (116 residues), which arises by limited autolysis of the 67-kDa native cucumisin (3, 5). The optimal pH and temperature of the caseinolytic activity of cucumisin were found to be 10.5 and around 70 °C, respectively (3), and its substrate specificity was reported to be fairly broad (6).

Since the cloning of the cucumisin cDNA, many other plant cDNAs for subtilisin-like serine proteases (subtilases) have been cloned. Subtilases constitute the SB family within the SB clan of serine proteases (7) and are subdivided into six families based on their sequence similarities. Most plant subtilases are grouped into the pyrolysin family, which is characterized by a large insertion between the stabilizing Asn and the reactive Ser and/or long COOH-terminal extensions (8). In Arabidopsis, 56 genes predicted to encode functional subtilases have been annotated (9). Plant subtilases are involved in many physiological processes, such as microsporogenesis, symbiosis, hypersensitive response, signal transduction and differentiation, senescence, and protein processing (for reviews, see Refs. 2 and 10). For instance, SDD1 and ALE1 are involved in stomatal development or cuticle formation and epidermal differentiation, respectively. AtSBT1.7 (also termed ARA12) is involved in the maturation of the seed coat (11), and AtSBT6.1 is implicated in

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Recombinant Propeptide Inhibits Mature Cucumisin

TABLE 1
Oligonucleotides used for amplification by PCR of cDNAs for full-length and six partial cucumisin propeptides

| Oligonucleotide | Nucleotide sequence (5' to 3') |
|-----------------|-------------------------------|
| P-1             | GGGGGCTAGGCAAGATTGAGTCTGACGA  |
| P-2             | GGGGGACTTTCTAGATGCTTCATTT    |
| P-3             | GGGGGCTAGCAACATTATTATATTAT    |
| P-4             | GGGGGCTAGGCGGGAGGAGCTGAGGA    |
| P-5             | GGGGGCTCTTTCTGAAACACGACACCA   |
| P-6             | GGGGGCTCTTGATCGATGTCCTGCTT    |
| P-7             | GGGGGCTCTGTTCTGATTGATCTGTA    |
| P-8             | GGGGGCTGTGTTGATTCTGACGTA      |
| P-9             | GGGGGCTGGCGCCAGGCTGTCATATA    |
| P-10            | GGGGGCTCTTAGCCAGAAAGCAGTACA   |
| P-11            | GGGGGCTCTCAATTCTCATGSCATAG    |
| P-12            | GGGGGCTCTCAATTCTCATGSCATAG    |
| P-13            | GGGGGCTCTCAATTCTCATGSCATAG    |

2 The abbreviations used are: ER, endoplasmic reticulum; cuc-pro, cucumisin propeptide; Glt, glutaryl; pNA, p-nitroanilide.

stress-induced processing of a membrane-associated transcription factor, thus inducing the expression of stress response gene (12).

Despite the prevalence and importance of plant subtilases, information on their enzyme activities and structures is very limited. Recently the x-ray diffraction analysis of a tomato subtilase (SBT3) has been reported (13). The primary structures of cucumisin and other plant subtilases suggest that they are secretory enzymes synthesized as inactive preproproteins and targeted to the endoplasmic reticulum (ER) by signal peptides. The NH2-terminal amino acid sequence of the mature enzyme, which was first analyzed for cucumisin, is conserved among most plant subtilases. Amino acids at positions +1 and +2 are both Thr, and those at positions +3 and +4 are Arg/His and Thr/Ser, respectively (14). This suggests a common mechanism for the propeptide processing of plant subtilase precursors. Detailed mechanisms of subsequent processing and activation of plant subtilase precursors are unknown except for a recent report demonstrating that the prodomain cleavage of a tomato subtilase 3 (SISBT3) occurs autocatalytically and that thezymogen maturation is an intramolecular process (15).

For bacterial and mammalian subtilases, much work has focused on the subsequent processing of the zymogens and its relevance for enzyme maturation. The prodomains of bacterial subtilisins are autocatalytically cleaved at their junction with the catalytic domains. They remain non-covalently bound and act as specific inhibitors of proteolytic activity (16, 17). Also, subtilisin propeptides can act as intramolecular chaperones assisting the correct folding of the mature enzyme (18, 19). Prodomain function and processing have also been investigated in detail for kinase-like mammalian proprotein convertases. For instance, the cleavage of the prodomain of furin at its junction with the catalytic domain occurs in a rapid intramolecular reaction in the ER, and this is necessary for the protein to fold into its native state (20). To date, however, no information is available on the roles of the prodomains of plant serine proteases, and the biochemical characterization of propeptides remains to be elucidated.

Here, we describe the strong inhibitory activity of the cucumisin propeptide against mature cucumisin and the relationship between the secondary structure and the inhibitory activity of the cucumisin propeptide. This is the first report demonstrating that the propeptide of a plant serine protease acts as a tightly binding competitive inhibitor of the mature enzyme and that the secondary structure of the propeptide is indispensable for its inhibitory activity.

EXPERIMENTAL PROCEDURES

Reagents—Restriction and modification enzymes were obtained from New England Biolabs, Roche Applied Science, and Promega Corp. Glutaryl-1-alamyl-1-alamyl-1-prolyl-1-leucine p-nitroanilide (Glt-Ala-Ala-Pro-Leu-pNA) was purchased from Peptide Institute (Osaka, Japan). All other commonly available reagents were of analytical grade.

Subcloning of cDNAs for Propeptides of Several Subtilases and Expression of Recombinant Peptides in E. coli—General DNA manipulations were carried out using standard procedures (21). Complementary DNAs for cucumisin, ARA12 (termed AtSBT1.7 in A. thaliana subtilase code), and RSP1 were described in our previous studies (5, 14). Subtilisin E cDNA was a gift from Dr. Hiroshi Takagi (22). Each cDNA was amplified by PCR using the cucumisin cDNA as a template and expressed in E. coli as His6-tagged proteins of the cucumisin propeptide, designated cuc-pro, and its short peptides, designated cuc-proΔN9, cuc-proΔN16, cuc-proΔC7, and cuc-proΔC14. The synthesized oligonucleotide primers are listed in Table 1. The primer sets used for PCR were as follows: P-1 and P-2 for cuc-pro, P-2 and P-3 for cuc-proΔN9, P-2 and P-4 for cuc-proΔN16, P-1 and P-5 for cuc-proΔC7, and P-1 and P-6 for cuc-proΔC14. After digesting the PCR products with Nhel and HindIII, the DNAs were subcloned into the corresponding restriction sites of pET28a (Merck) and introduced into E. coli Rosetta (DE3) (Merck). The nucleotide sequences of the resulting subclones were confirmed on both strands by sequencing using an automated sequencer (model 4000L, LI-COR Biosciences, Inc., Lincoln, NE). For the expression of wild-type cucumisin propeptide (cuc-pro-WT) that has no extra amino acids in the NH2 terminus, such as Hisα-tag, the nucleotide sequence was amplified using the primers P-7 and P-2 after which it was ligated into Ncol-HindIII sites of pET28a. For CDNA amplification of three propeptides, ARA12, RSP1, and subtilisin E, the primer sets used were P-8 and P-9, P-10 and P-11, and P-12 and P-13, respectively. Each PCR product was ligated into BamHI-HindIII, Nhel-HindIII, and Nhel-HindIII sites in pET28a, respec-
Recombinant Propeptide Inhibits Mature Cucumisin

To express recombinant proteins, transformed cells were cultured in LB medium containing 50 μg/ml kanamycin at 37 °C until an absorbance of 0.6 at 600 nm was reached. Recombinant proteins were induced by adding 1 mM isopropyl β-D-thiogalactopyranoside for 16 h at 37 °C.

Site-directed Mutagenesis of Recombinant Cucumisin Propeptide—Site-directed mutagenesis was used to introduce amino acid substitutions using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. Oligonucleotide primers used for the site-directed mutagenesis are listed in Table 2. All cDNA sequences used for mutated propeptides were verified by DNA sequencing.

Purification of Recombinant Propeptide—Purification of recombinant propeptides was performed at 4 °C. Transformed cells were harvested by centrifugation at 8,000 × g for 10 min, suspended in buffer A (50 mM sodium phosphate buffer, pH 7.5 containing 0.3 M NaCl and 5 mM β-mercaptoethanol), and homogenized with a supersonic wave using a UD-200 ultrasonic disruptor (TOMY Co. Ltd., Tokyo, Japan) with 30 min, and insoluble debris were removed by centrifugation at 12,000 × g for 20 min. The supernatant was filtered through a 0.45-μm nitrocellulose membrane filter (ADVANTEC Co. Ltd., Tokyo, Japan). The protein solution was put on a CM-Sepharose Fast Flow (GE Healthcare) column (1.6-cm inner diameter × 10 cm) equilibrated with the same buffer. After washing the column with buffer B, the protein was eluted stepwise with every 5 ml of 25–150 mM NaCl in the same buffer. The yields of wild-type and mutant propeptides were 30 and 70–90%, respectively. The fractions containing recombinant proteins were pooled and used for further analysis.

Purification of Cucumisin—Prince melons (C. melo L. cv. Prince) were cultivated at the experimental farm attached to the Faculty of Agriculture, Kobe University from April to August. Fruits were tagged upon pollination, and developing fruits were harvested between 15 and 20 days after pollination. Purification of cucumisin was performed at 4 °C as described previously (5) with slight modification. The central parts of the fruits were separated from the sarcocarp and washed with buffer E (50 mM sodium acetate buffer, pH 5.0 containing 0.3 M NaCl). This wash was combined with the juice from the central parts of the fruits and used as the crude extract. After centrifugation at 8,000 × g for 15 min, solid ammonium sulfate was added to the supernatant to 60% saturation, and the precipitate was collected by centrifugation at 12,000 × g for 20 min. The proteins were dissolved in a small volume of buffer E and put on a HiPrep 16/60 Sephacryl S-200 HR column (1.6-cm inner diameter × 60 cm; GE Healthcare) equilibrated with the same buffer. The eluted fractions containing protease activity were pooled and precipitated with 60% saturated ammonium sulfate. The proteins were dissolved in a minimum volume of buffer E and put on a HiPrep 16/60 Sephacryl S-200 HR column (1.6-cm inner diameter × 60 cm; GE Healthcare) equilibrated with the same buffer. The eluted fractions containing protease activity were pooled and precipitated with 60% saturated ammonium sulfate. The proteins were dissolved in a minimum volume of buffer E, and gel filtration using the HiPrep 16/60 Sephacryl S-200 HR column was repeated in the same manner. The protease fractions were collected by ammonium sulfate precipitation, dissolved in a small volume of buffer F, and dialyzed against the same buffer for 16 h. The protease solution was put on a CM-Sepharose Fast Flow (GE Healthcare) column (1.6-cm inner diameter × 10 cm) equilibrated with the same buffer. After washing with buffer F, the protease was eluted in the same buffer with a liner gradient of 0–200 mM NaCl. The protease fractions that were eluted as a single peak were pooled and precipitated with ammonium sulfate. The pre-

### Table 2

Oligonucleotides used for cucumisin propeptide mutagenesis

| Oligonucleotide sequence (5’ to 3’) | Mutants |
|-------------------------------------|---------|
| GATGATGGGAAAACCTTATATAGGATACG     | I33A    |
| CCAGCTCTATACAAATGAGGCACTACG       | I35A    |
| GAAAACATATTGCTGGATATACGTTG        | I35A    |
| CCCCAGGTCTAATTTGCATTTTTTTTTC      | V36A    |
| CATTTATATGGATACGAGGGAAGGAG        | V36A    |
| CTCCTCTGACCCATCTACTACAGAATATAG   | V36T    |
| GAAAACATATTGCTGGGAGGCATTG         | Y37A    |
| CTTTCTCCTCATCATCTCATTCTAAAAAGG    | Y37A    |
| GAGGTGCGCTTCCTATTCTG               | Y37A    |
| CATTCTTTAACGACACGCAACAACCCTC      | I35A/V36A/Y37A |
| GAGGTGCGCTTCTG                 | I35A/V36A/Y37A |
| CATTCTTTAACGACACGCAACAACCCTC      | Y98A    |
| GAGGTGCGCTTCTG               | V101A   |
| CATTCTTTAACGACACGCAACAACCCTC      | V101A   |
| GAGGTGCGCTTCTG               | V101A   |
| CATTCTTTAACGACACGCAACAACCCTC      | V98A/V101A |
| GAGGTGCGCTTCTG               | V98A/V101A |
Recombinant Propeptide Inhibits Mature Cucumisin

Although the amino acid sequences of plant subtilase propeptides have weak similarities with that of bacterial subtilisin and several hydrophobic amino acids are well conserved between plant subtilases and bacterial subtilisin as described above, it was assumed that plant subtilase propeptides have functions similar to those of the subtilisin propeptide. To evaluate this assumption, cDNAs for the propeptides of cucumisin, Arabidopsis ARA12, rice RSP1, subtilisin E, and several cucumisin propeptide mutants were subcloned into expression vectors in E. coli as His-tagged peptides with 23 extra amino acids at the NH$_2$ terminus. Then, the inhibitory activities of purified recombinant propeptides against mature cucumisin were assayed. As all of the recombinant proteins were insoluble in aqueous solution, they were dissolved in a buffer containing 8 M urea and then purified by affinity column chromatography using a nickel-Sepharose column after which the urea was removed by dialysis as described under “Experimental Procedures.” Because the cucumisin propeptide without extra amino acids at the NH$_2$ terminus (cuc-pro-WT) expressed in E. coli was also insoluble, it was dissolved in the urea-containing buffer and then dialyzed to remove urea after which an additional DEAE-Sepharose column chromatography step was carried out for thorough purification. The homogeneities of the purified propeptides were confirmed by SDS-PAGE. Each purified recombinant propeptide migrated as a single major protein band (Fig. 2).

Inhibitory Activities of Recombinant Cucumisin Propeptide and Related Polypeptides—Recombinant cuc-pro-WT strongly inhibited cucumisin (Fig. 3A). Preincubation of cucumisin with cuc-pro-WT for 10 min before the enzyme assay did not affect the remaining activity of cucumisin (data not shown), suggesting that the propeptide acted as a typical rapid equilibrium inhibitor. Using 16 nM cucumisin and 2.1 mM Glu-Ala-Ala-Pro-Leu-pDNA as a substrate, the IC$_{50}$ value for inhibition by cuc-pro-WT was 20 nM, and the $K_I$ value determined was 6.2 ±
0.55 nm (Fig. 3A). These results indicated that the cucumisin propeptide was a tightly binding inhibitor of cucumisin. As shown in Fig. 3B, the IC50 values for inhibition of cucumisin by cuc-pro-WT increased linearly in proportion to the substrate concentration, clearly demonstrating a competitive type of inhibition.

As some subtilase propeptides have been shown to inhibit not only their cognate proteases but also other homologous proteases (27), we examined whether the propeptides of two plant subtilases, ARA12 and RSP1, and subtilisin E could inhibit mature cucumisin. Interestingly, ARA12 and RSP1 propeptides inhibited cucumisin with the Ki values of 62.0/11006 and 100/11006 nM, respectively, but that of subtilisin E did not inhibit cucumisin (Fig. 3C). In contrast, subtilisin Carlsberg was not inhibited by the propeptides of three plant subtilases (cucumisin, ARA12, and RSP1) but was strongly inhibited by the subtilisin E propeptide as reported previously (data not shown) (18). These results suggested the compatibility with and the inhibitory specificity of plant subtilase propeptides for their cognate enzymes.

Important Region(s) within Cucumisin Propeptide for Inhibition of Mature Enzyme—To evaluate the important region(s) within the cucumisin propeptide for the inhibition of mature enzyme, we expressed NH2- or COOH-terminal truncated propeptides in E. coli as His6-tagged proteins and measured the inhibitory activities of these recombinant propeptides against mature cucumisin (Fig. 4). The 9 NH2-terminal amino acid-truncated propeptide (cuc-proN9) still had strong inhibitory activity against mature cucumisin (Ki = 14.7 ± 0.72 nM) comparable with that of cuc-pro (Ki = 7.1 ± 0.37 nM), whereas a 16-amino acid-truncated propeptide (cuc-proN16) showed a much weaker inhibition (Ki = 5.5 ± 0.73 M). This suggested that the region from Asn32 to Met38 (NIYIVYM) was important for the inhibitory activity. Similarly, the 7 COOH-terminal amino acid-deleted propeptide (cuc-proC7) showed strong inhibition (Ki = 52.6 ± 6.5 nM) (i.e. about 6 times less inhibition than cuc-pro), whereas the 14 COOH-terminal amino acid-deleted propeptide (cuc-proC14) resulted in no inhibition against cucumisin, suggesting that the region from Gly97 to Leu103 (GVVSVFL) was also important for the inhibitory activity. It should be noted that these two important regions, NIYIVYM and GVVSVFL, have hydrophobic characteristics. Collectively, these results indicate that each Asn32–Met38 and Gly97–Leu103 region has no inhibitory activity by itself, and the possible cooperation between these two hydrophobic regions is likely necessary for the inhibitory activity of the propeptide.

Inhibitory Activities of Point Mutants Derived from Cucumisin Propeptide—The hydrophobic amino acid residues Ile35, Val36, Tyr37, Val98, and Val101 in Asn32–Met38 and Gly97–Leu103 regions within the cucumisin propeptide are well conserved among plant subtilases (Fig. 1). Hydrophobic amino acids Val41, Val94, and Val97 in the subtilisin E propeptide, co-
responding to Val^{36}, Val^{98}, and Val^{101} in the cucumisin propeptide, were reported to form a hydrophobic core (26, 28). As it was expected that these hydrophobic residues in the cucumisin propeptide would be responsible for the inhibitory activity against cucumisin, site-directed mutagenesis of these residues for substitution to Ala were performed as described under “Experimental Procedures.” The inhibitory activities of these recombinant propeptide mutants were measured. In addition, Ile^{33}, located within the Asn^{32}–Met^{38} region but not a conserved hydrophobic residue among plant subtilases, was also substituted with Ala. The $K_i$ values of these mutants against cucumisin are listed in Fig. 4. The mutants I33A ($K_i = 7.4 \pm 0.79 \text{ nM}$), I35A ($9.2 \pm 0.68 \text{ nM}$), and Y37A ($9.0 \pm 0.85 \text{ nM}$) showed strong inhibitions comparable with cuc-pro. The mutant V36A, however, showed 5 times less inhibition ($K_i = 37.4 \pm 4.0 \text{ nM}$) than cuc-pro. These results indicate that Val^{36} is the most important amino acid in the Asn^{32}–Met^{35} hydrophobic region for the inhibitory activity and that Ile^{35} and Tyr^{37} contribute to the inhibitory activity of the propeptide cooperatively with Val^{36} by forming an extended hydrophobic cluster around Val^{36}. The Ala substitution of Val^{98} or Val^{101} within the Gly^{97}–Leu^{103} region had no significant effect on the inhibitory activity. The double Ala substitution mutant, V98A/V101A, reduced the inhibitory activity ($K_i = 21 \pm 0.56 \text{ nM}$) but to a lesser extent. The triple Ala substitution mutant, I35A/V36A/Y37A, resulted in a further loss of the inhibitory activity ($K_i = 200 \pm 27 \text{ nM}$). However, another triple Ala substitution mutant,
V36A/V98A/V101A, showed only slightly weaker inhibition than V36A. These results suggest that Val98 and Val101, which are conserved among many subtilases, are not too important for the inhibition and the possible cooperation between two hydrophobic regions.

CD Spectroscopy for Cucumisin Propeptide—Recombinant subtilisin propeptide is completely unfolded without a protease domain and is folded when it binds to the protease domain (29, 30), although some subtilase propeptides are folded without protease domains (27, 31). To determine whether the recombinant cucumisin propeptide had a stable conformation without a protease domain, CD spectroscopy was performed. Under non-denaturing conditions, the spectra of cuc-pro and cuc-pro-WT were very similar to each other and revealed some negative ellipticity at 208 and 222 nm (Fig. 5A), corresponding to the CD spectrum of random coil and $\alpha$-helix, respectively. $\beta$-Sheet also seems to contribute to the spectrum around 215 nm. In contrast, the intensity around 215–230 nm remarkably decreased under denaturing conditions with 8M urea, indicating the decrease of ordered secondary structures except for random coil. The deconvolution of the CD spectra suggests that cuc-pro-WT and cuc-pro contain 15.0 and 15.9% $\alpha$-helix and 33.3 and 33.5% $\beta$-sheet, respectively (Table 3). These results indicate that both cucumisin propeptides, with or without 23 extra NH2-terminal amino acids, have secondary structures by themselves without the protease domain. As there were no significant differences of the $K_i$ values between cuc-pro (7.1 ± 0.37 nM) and cuc-pro-WT (6.2 ± 0.55 nM), the 23 NH2-terminal amino acids in cuc-pro were likely not to affect either the inhibitory activity or the secondary structure of the cucumisin propeptide. This validated the use of His6-tagged recombinant propeptides, such as cuc-pro and its mutants, for the following experiments.

Thermal Stability of Cuc-pro Conformation—For cuc-pro, the intensity of the negative ellipticity at 222 nm in its CD spectrum decreased with increasing temperature from 30 to 90 °C, indicating the unfolding of cuc-pro by heat treatment (Fig. 5B). The transition temperature ($T_m$) for thermal unfolding was ~55 °C. In contrast, when the temperature decreased from 90 to 30 °C, the intensity of the negative ellipticity increased and returned to its level before heating (Fig. 5C). The intensity of the negative ellipticity at 222 nm also decreased with increasing urea concentration at 30 °C (Fig. 5D). The decrease of intensity in 8 M urea, however, was significantly larger than that at 90 °C without urea (Fig. 5B and D). These results suggested that the thermal unfolding of the cucumisin propeptide, even at 90 °C, was partial and reversible. The inhibitory activity of cuc-pro was also fairly stable up to 90 °C for 10 min (Fig. 5E). Because the inhibition assay was performed at 30 °C, the structure of cuc-pro was thought to have quickly recovered during the enzyme assay, demonstrating the reversibility of the thermal unfolding of the cucumisin propeptide.

CD Spectroscopy for Ala Substitution Mutants—If the tripeptide Ile35-Val36-Tyr37 formed a hydrophobic core within the cucumisin propeptide, Ala substitution of these residues could affect the propeptide conformation. To verify this hypothesis, CD spectra of Ala substitution mutants were measured. The CD spectrum of the Y37A mutant, which strongly inhibited cucumisin, was very similar to that of cuc-pro (Fig. 6). In contrast, the CD spectra of V36A and I35A/V36A/Y37A revealed that the ordered secondary structures of these mutants were significantly decreased. In particular, the decreasing intensity of the negative ellipticity at 222 nm was remarkable and correlated with the reduction of the inhibitory activity. The estimated content of $\alpha$-helix in these
two mutants was also significantly lower than that in cuc-pro, especially in I35A/V36A/Y37A (9.3%) (Table 3). These results suggest that Ile35, Val36, and Tyr37 contribute to the formation of the proper secondary and, probably, the tertiary structure necessary to inhibit cucumisin.

**DISCUSSION**

Plant subtilases are thought to be synthesized as precursors containing N-terminal propeptides, but the functions of these propeptides are unknown. We found that purified recombinant cucumisin propeptide is a potent tightly binding competitive inhibitor of mature cucumisin. The $K_i$ value of cuc-pro-WT was $6.2 \pm 0.55 \text{ nM}$, suggesting that the enzymatic activity of the plant subtilase zymogen was regulated by the strong inhibitory activity of its propeptide. In this regard, the propeptides of bacterial subtilisin have also been reported to act as competitive inhibitors of their protease domains with inhibition constants in the nanomolar range (17, 18). Besides plant subtilases, the propeptides of plant thiol proteases, such as papain and papaya proteinase IV, were also reported to inhibit their cognate proteases (32, 33).

The proteolytic activity of cucumisin was also strongly inhibited by ARA12 and RSP1 propeptides but not by the subtilisin E propeptide (Fig. 3C). In contrast, the propeptides of cucumisin, ARA12, and RSP1 did not inhibit bacterial

![FIGURE 5](image-url)  
**FIGURE 5.** *Structural characterization of recombinant cucumisin propeptide.* A, far-UV CD spectra of cuc-pro-WT (solid gray line), cuc-pro (solid black line), and cuc-pro in 8 M urea (black dashed line). B and C, reversibility of structural changes caused by heat treatment. Structural changes of cuc-pro monitored by negative ellipticity at 220 nm are dependent on increasing (B) or decreasing (C) temperature. The $T_m$ of cuc-pro is $55 \degree C$. D, structural changes of cuc-pro monitored by negative ellipticity at 220 nm are dependent on urea concentration. Data shown represent mean values obtained from duplicate experiments. E, thermal stability of the inhibitory activity of cuc-pro. Cuc-pro was incubated at different temperatures for 10 min, and then the remaining inhibitory activity was measured at 30 $\degree C$. Data shown represent mean values obtained from six independent experiments, and the error bars indicate the S.E. deg, degrees.

![FIGURE 6](image-url)  
**FIGURE 6.** *CD spectra of Ala substitution mutants of cucumisin propeptide.* Far-UV CD spectra of cuc-pro (black solid line), I35A (gray solid line), V36A (gray dot line), Y37A (gray dashed line), and I35A/V36A/Y37A (black dashed line) are shown. deg, degrees.

![TABLE 3](image-url)  
**TABLE 3**

Secondary structure content of cuc-pro and four Ala substitution mutants  
Secondary structure contents were calculated using the CONTIN/ll algorithm (25).

| Propeptides               | $\alpha$-Helix | $\beta$-Sheet |
|---------------------------|----------------|--------------|
| Cuc-pro-WT                | 15.0           | 33.3         |
| Cuc-pro                   | 15.9           | 33.5         |
| Cuc-pro in 8 M urea       | 8.0            | 11.5         |
| I35A                      | 15.8           | 31.1         |
| V36A                      | 14.4           | 32.1         |
| Y37A                      | 17.0           | 32.1         |
| I35A/V36A/Y37A            | 9.3            | 33.0         |
Recombinant Propeptide Inhibits Mature Cucumisin

subtilisin. The amino acid sequences of ARA12 and RSP1 propeptides are about 36% identical to that of the cucumisin propeptide (Fig. 1), and the K_i values of cucumisin inhibition by ARA12 and RSP1 propeptides were about 20-fold higher than that of the cucumisin propeptide. These results show that the inhibitory activities of plant subtilase propeptides are dependent on their selectivity and compatibility with their cognate enzymes. In this regard, it has also been reported that propeptides of some bacterial subtilases inhibit other types of subtilase. For example, the aqualysin I propeptide, a thermostable subtilase synthesized by *Thermus aquaticus* YT-1, inhibits not only aqualysin I but also subtilisin BPN’ (27). The amino acid sequence of the aqualysin I propeptide, however, is only 21% identical to that of the subtilisin E propeptide. In another astonishing example, *Pleurotus ostreatus* proteinase A inhibitor 1 (POIA1), which is not a protease propeptide, can inhibit subtilisin BPN’ and can act as its intramolecular chaperone, although the amino acid sequence of POIA1 is only 18% identical to that of the subtilisin BPN’ propeptide (34). Despite the low amino acid sequence similarity between POIA1 and subtilisin BPN’ propeptides, the overall structural topology of the POIA1 propeptide is very similar to that of the subtilisin BPN’ propeptide (35). These findings strongly support the idea that higher order structures of plant subtilases propeptides, rather than their primary structures, are important for their inhibitory activities against their cognate enzymes.

Many bacterial subtilases are activated by removing their propeptides by autoprocessing, and the COOH termini of the propeptides are thought to inhibit enzymes by binding to their propeptide sequences (36). Clarifying the site and timing of the processing of the prodomain of plant subtilase precursor, Cedzich et al. (15) recently reported that the cleavage of the prodomain of tomato SISBT3 occurs autocatalytically, and zymogen maturation is an intramolecular process.

The site of the processing of cucumisin prodomain and the conditions under which the propeptide-enzyme complex dissociates in planta are unknown. It has been reported that the processing of the prodomain of tomato SISBT3 in the ER is a prerequisite for passage through the secretory pathway using a transient expression system in *Nicotiana benthamiana* leaves (15). As cucumisin is secreted and accumulated in the juice in melon fruits, it is also likely to be sorted along the secretory pathway after processing of the prodomain in the ER. Regarding the activation of the propeptide-enzyme complex, pH-regulated activation of furin in the secretory pathway and a pH sensor in the furin propeptide have been reported (36). Clarifying the site and timing of the processing of cucumisin prodomain and the mechanisms of the dissociation of propeptide-enzyme complexes will be major tasks for the future.

The NH2-terminal amino acid residues of mature regions (Thr-Thr-(Arg/His)-Ser/Thr) are well conserved among plant subtilases (Fig. 1) (14). Regardless of the sequence homologies of NH2-terminal amino acid residues of mature plant subtilases, the substrate specificities of plant subtilases that have so far been reported were quite different from each other. For example, cucumisin shows broad substrate specificity; ARA12 shows preference for Phe and Ala at the P1 position and for Asp, Leu, and Ala at the P1’ position (37); soybean C1 prefers Gln at the P1 and Gln/Gln at the P1’ position (38); and tomato SISBT3 shows a preference for Gln and Lys at P1 and P2 positions (15). These findings suggest that the mechanisms for recognizing the propeptide-processing site are different from that for substrate recognition during proteolysis by mature proteases.

The analysis of the important region(s) within the cucumisin propeptide for the inhibition of the mature enzyme using recombinant truncated propeptides and Ala-substituted mutants (Fig. 4) clearly showed that each of the two hydrophobic regions, Asn32–Met38 (NIYIVYM) and Gly97–Leu103 (GVVSVFL), had no inhibitory activity by itself. Thus, the possible cooperation between these two hydrophobic regions along with the formation of the higher order structure is likely necessary for the inhibitory activity of the propeptide. Indeed, the CD spectrum of the cucumisin propeptide revealed that it has a secondary structure by itself without the protease domain (Fig. 5A). In this respect, the propeptides of aqualysin I (27), PisSUB-1 (31), and human proprotein convertases (39) were also reported to form secondary structure by themselves. By comparison, the subtilisin BPN’ propeptide has been reported to be unfolded by itself and is folded correctly only when it forms a complex with the protease domain (28, 30). The structure of cucumisin prodomain also may be changed to some extent upon binding to the protease domain. Interestingly, the aqualysin I propeptide can inhibit subtilisin BPN’ more strongly than the subtilisin BPN’ propeptide (27). The mutants of subtilisin BPN’ propeptide, which could have some secondary structures because of the introduction of amino acid replacements, had K_i values of inhibition against subtilisin BPN’ lower than that of a wild-type propeptide (30). These studies on bacterial subtilisins also support the idea that formation of the secondary structure is necessary for the inhibitory activity of the cucumisin propeptide against cucumisin.

Cuc-pro and Y37A had very similar CD spectra, suggesting that an Ala substitution at Tyr37 did not affect the secondary structure of the cucumisin propeptide (Fig. 6). In contrast, the CD spectra of V36A and I35A/V36A/Y37A were different from that of cuc-pro especially with regard to the noticeable decreasing intensity of negative ellipticity at 222 nm, suggesting that the content of the ordered secondary structure in V36A and I35A/V36A/Y37A was decreased after the substitutions to Ala. The decreasing intensity of negative ellipticity at 222 nm for the ordered secondary structure of I35A/V36A/Y37A was more remarkable than that of V36A. K_i values of both V36A (37.4 ± 4.0 nM) and I35A/V36A/Y37A (200 ± 27 nM) were higher than...
Recombinant Propeptide Inhibits Mature Cucumisin

that of cuc-pro (7.1 ± 0.37 nm), and I35A/V36A/Y37A inhibited cucumisin more weakly than did V36A (Fig. 4). The CD spectrum of I35A was also different from that of cuc-pro as the intensity of the negative ellipticity around 203 nm for the secondary structure of I35A was remarkably increased, similar to V36A (Fig. 6). For the CD spectrum around 222 nm, however, I35A was more similar to cuc-pro than to V36A. Because I35A had a strong inhibitory activity comparable with cuc-pro, the change of the secondary structure monitored around 203 nm for the Ala substitution of Ile45, which shows the increase of random coiled structure, was not likely critical for the inhibitory activity. As shown in Table 3, the estimated content of α-helix in V36A (14.4%) was lower than that of cuc-pro (15.9%). The decrease of the α-helix content in I35A/V36A/Y37A (9.3%) was remarkable. Collectively, the secondary structure monitored around 215–222 nm, primarily due to the contribution of α-helices and β-sheets, is suggested to be important for the inhibition of the protease domain. The random coiled structure observed for cuc-pro and its mutants may be converted to ordered secondary structures after docking to the cognate protease.

We described that the proper secondary structure along with the assistance of some hydrophobic residues was evidently important for the inhibitory activity of cucumisin propeptides. For the maturation of the cucumisin precursor, disabling the inhibitory activity of the propeptide and its degradation by the cognate or other protease activity prior to the activation of the cucumisin precursor were thought to be essential. The analyses of NH2- or COOH-terminal truncated mutants suggested that the degradation of the two hydrophobic regions in the cucumisin propeptide could easily weaken its inhibitory activity. To evaluate the detailed mechanisms of the inhibition by the propeptides of cucumisin and other plant subtilases, further structural studies, including x-ray analysis, will be necessary.

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REFERENCES

1. Beers, E. P., Jones, A. M., and Dickerman, A. W. (2004) Phytochemistry 65, 43–58
2. Schaller, A. (2004) Planta 220, 183–197
3. Yamagata, H., Ueno, S., and Iwasaki, T. (1989) Agric. Biol. Chem. 53, 1009–1017
4. Yamagata, H., Yonesu, K., Hirata, A., and Aizono, Y. (2002) J. Biol. Chem. 277, 11582–11590
5. Yamagata, H., Masuzawa, T., Nagoaka, Y., Ohnishi, T., and Iwasaki, T. (1994) J. Biol. Chem. 269, 32725–32731
6. Uchikoba, T., Yonezawa, H., and Kaneda, M. (1995) J. Biochem. 117, 1126–1130
7. Rawlings, N. D., Morton, F. R., Kok, C. Y., Kong, J., and Barrett, A. J. (2008) Nucleic Acids Res. 36, D320–D325
8. Siezen, R. J., and Leunissen, J. A. (1997) Protein Sci. 6, 501–523
9. Rautengarten, C., Steinhauser, D., Büsis, D., Stintzi, A., Schaller, A., Kopka, J., and Altmann, T. (2005) PLoS Comput. Biol. 1, e40
10. Antão, C. M., and Malcata, F. X. (2005) Plant Physiol. Biochem. 43, 637–650
11. Rautengarten, C., Usadel, B., Neumetzler, L., Hartmann, J., Büsis, D., and Altmann, T. (2008) Plant J. 54, 466–480
12. Liu, J. X., Srivastava, R., Che, P., and Howell, S. H. (2007) Plant J. 51, 897–909
13. Ottmann, C., Rose, R., Huttonlocher, F., Cedzich, A., Hauske, P., Kaiser, M., Huber, R., and Schaller, A. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 17223–17228
14. Yamagata, H., Uesugi, M., Saka, K., Iwasaki, T., and Aizono, Y. (2000) Biosci. Biotechnol. Biochem. 64, 1947–1957
15. Cedzich, A., Huttonlocher, F., Kuhn, B. M., Pfannstiel, J., Gabler, L., Stintzi, A., and Schaller, A. (2009) J. Biol. Chem. 284, 14068–14078
16. Baker, D., Silen, J. L., and Agard, D. A. (1992) Proteins 12, 339–344
17. Huang, H. W., Chen, W. C., Wu, C. Y., Yu, H. C., Lin, W. Y., Chen, S. T., and Wang, K. T. (1997) Protein Eng. 10, 1227–1233
18. Li, Y., Hu, Z., Jordan, F., and Inouye, M. (1995) J. Biol. Chem. 270, 25127–25132
19. Takagi, H., Koga, M., Katsurada, S., Yabuta, Y., Shinde, U., Inouye, M., and Nakamori, S. (2001) FEBS Lett. 508, 210–214
20. Anderson, E. D., Molloy, S. S., Jean, F., Fei, H., Shimamura, S., and Thomas, G. (2002) J. Biol. Chem. 277, 12879–12890
21. Sambrook, J., and Russell, D. W. (2001) Molecular Cloning A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
22. Ikemura, H., Takagi, H., and Inouye, M. (1987) J. Biol. Chem. 262, 7859–7864
23. Walker, I. M. (ed) (2005) The Proteomics Protocols Handbook, pp. 571–607, Humana Press, Totowa, NJ
24. Copeland, R. A. (2000) Enzymes, 2nd Ed., pp. 76–145, Wiley-VCH, New York
25. Srerama, N., and Woody, R. W. (2000) Anal. Biochem. 287, 252–260
26. Shinde, U., Xu, F., and Inouye, M. (1999) J. Biol. Chem. 274, 15615–15621
27. Marie-Claire, C., Yabuta, Y., Suefuji, K., Matsuzawa, H., and Shinde, U. (2001) J. Mol. Biol. 305, 151–165
28. Jain, S. C., Shinde, U., Li, Y., Inouye, M., and Berman, H. M. (1998) J. Mol. Biol. 284, 137–144
29. Eder, L., Rheinheimer, M., and Fersht, A. R. (1993) J. Mol. Biol. 233, 293–304
30. Kojima, S., Minagawa, T., and Miura, K. (1998) J. Mol. Biol. 277, 1007–1013
31. Jean, L., Hackett, F., Martin, S. R., and Blackman, M. J. (2003) J. Biol. Chem. 278, 28572–28579
32. Taylor, M. A., Baker, K. C., Briggs, G. S., Connerton, I. F., Cummings, N. J., Pratt, K. A., Revell, D. F., Freedman, R. B., and Goodenough, P. W. (1995) Protein Eng. 8, 59–62
33. Groves, M. R., Coulombe, R., Jenkins, J., and Cysler, M. (1998) Proteins 32, 504–514
34. Kojima, S., Ichihara, A., and Yanai, H. (2005) FEBS Lett. 579, 4430–4436
35. Sakawaka, H., Yoshinaga, S., Kojima, S., and Tamura, A. (2002) J. Biol. Chem. 307, 159–167
36. Feliciangeli, S. F., Thomas, L., Scott, G. K., Subbian, E., Hung C. H., Molloy, S. S., Jean, F., Shinde, U., and Thomas, G. (2006) J. Biol. Chem. 281, 16108–16115
37. Hamilton, J. M., Simpson, D. J., Hyman, S. C., Ndimba, B. K., and Slabas, A. R. (2003) Biochem. J. 370, 57–67
38. Boyd, P. M., Barnaby, N., Tan-Wilson, A., and Wilson, K. A. (2002) Biochim. Biophys. Acta 1596, 269–282
39. Fugère, M., Limperis, P. C., Beaulieu-Audy, V., Gagnon, F., Lavigne, P., Klarskov, K., Leduc, R., and Day, R. (2002) J. Biol. Chem. 277, 7468–7476
40. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007) Bioinformatics 23, 2947–2948
41. Emanuelsson, O., Brunak, S., von Heijne, G., and Nielsen, H. (2007) Nat. Protoc. 2, 953–971
