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FUNCTIONAL ANALYSIS OF THE CUCUMISIN PROPEPTIDE AS A POTENT INHIBITOR OF ITS MATURE ENZYME*

Masataka Nakagawa[1], Megumi Ueyama[1], Hiroki Tsuruta[2], Tomohide Uno[1], Kengo Kanamaru[1], Bunzo Mikami[3], and Hiroshi Yamagata[1]

From the [1]Laboratory of Biochemistry, Graduate School of Agricultural Science and [2]Office of Collaborative Research and Technology Development, Kobe University, Nada, Kobe 657-8501 and [3]Laboratory of Applied Structural Biology, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan

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Address correspondence to: Hiroshi Yamagata, Laboratory of Biochemistry, Graduate School of Agricultural Science, Kobe University, Rokkodai-cho 1-1, Nada-ku, Kobe, 657-8501 JAPAN. Tel & Fax: +81-78-803-5875, E-mail: yamagata@kobe-u.ac.jp

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 preproprotein consisting of a signal peptide, NH_2-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* independent of their mature enzymes. The cucumisin propeptide strongly inhibited cucumisin in a competitive manner with a K_i 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, Asn^{32} to Met^{38} and Gly^{97} to Leu^{103}, in the cucumisin propeptide were important for its inhibitory activity. Site-directed mutagenesis also confirmed the role of a Val^{36}-centerd hydrophobic cluster within the Asn^{32} to Met^{38} region in cucumisin inhibition. Circular dichroism spectroscopy revealed that the cucumisin propeptide had a secondary structure without a cognate protease domain, and that the thermal unfolding of the propeptide at 90°C was only partial and reversible. A tripeptide, $Ile^{35}Val^{36}Tyr^{37}$, in the Asn^{32} to Met^{38} region was thought 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), NH_2-terminal pro-sequence (88 residues), 54-kDa protease domain (505 residues), which is the active enzyme domain of the 67-kDa native cucumisin, 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 S8 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 C-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 (2,10) (see reviews 2, 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 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 ER by signal peptides. The N-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 (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 (SlSBT3) occurs autocatalytically and that the zymogen 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 kexin-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 tight-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 Inc., Boehringer Mannheim Co. Ltd. and Promega Co. Ltd. Glutaryl- L- alanyl- L- alanyl- L- prolyl- L-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 *Arabidopsis thaliana* subtilase code), and *RSP1* were described in our previous papers (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 $(His)_6$-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 the following: 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 *Nhe*I and *Hin*dIII, the DNAs were subcloned into the corresponding restriction sites of pET28a (Merck, Darmstadt, Germany) 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; LICOR, Inc., NE). For the expression of wild-type cucumisin propeptide (cuc-pro-WT) that has no extra amino acids in the N-terminal such as $(His)_6$-tag, the nucleotide sequence was amplified using the primers P-7 and P-2 after which they were ligated into *Nco*I-*Hin*dIII 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 *Bam*HI-*Hin*dIII, *Nhe*I-*Hin*dIII, and *Nhe*I-*Hin*dIII sites in pET28a, respectively. 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 the 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 Propeptides —— 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 Na-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) with output 2 for a total 5 min on ice. After centrifugation at 12,000 × *g* for 30 min, the pellet was solubilized in buffer B (buffer A containing 8 M Urea and 20 mM imidazole) with sonication. The solution was incubated for 16 h at 4°C. Insoluble debris was removed by centrifugation at 12,000 × *g* for 30 min, and the supernatant was filtered through a 0.45 μm nitrocellulose membrane filter (ADVANTEC Co. Ltd., Tokyo). The $(His)_6$-tagged propeptides were purified using a Ni-Sepharose HP and (HiTrap Chelating HP) column (ϕ 0.7 × 2.5 cm, GE Healthcare Bio-Sciences Co., Ltd., NJ) equilibrated with buffer B. After washing the column with buffer B, the protein was eluted stepwise with every 5 ml of 50 to 200 mM imidazole in the same buffer. The fractions containing recombinant proteins confirmed by 15% SDS-PAGE were pooled. The concentration of purified proteins was adjusted to about 50 μg/ml with buffer B, and the solution was dialyzed against buffer C (50 mM Na-phosphate buffer, pH 7.5, containing 0.2 M NaCl) for 24 h. To purify the cucumisin propeptide without the $(His)_6$-tag (cuc-pro-WT), the propeptide was solubilized in buffer B and dialyzed against buffer D (50 mM Na-phosphate buffer, pH 7.5) for 16 h. After

centrifugation at 12,000 × *g* for 30 min, the supernatant was filtered through a 0.45 μm nitrocellulose membrane filter, and put on a DEAE Sepharose column (ϕ 0.5 × 5 cm) equilibrated with buffer D. After washing the column with buffer D, the protein was eluted stepwise with every 5 ml of 25 to 150 mM NaCl in the same buffer. The yields of wild 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 (*Cucumis 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 to 20 days after pollination. Purification of cucumisin was performed at 4°C as described previously with slight modification (5). The central parts of the fruits were separated from the sarcocarp and washed with buffer E (50 mM Na-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 (ϕ 1.6 × 60 cm, GE Healthcare) equilibrated with the same buffer. The eluted fractions containing protease activity were pooled and precipitated with 60% saturation of ammonium sulfate. The proteins were dissolved in a minimum volume of buffer E, and gel filtration using 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 (50 mM Na-acetate buffer, pH 5.0), and dialyzed against the same buffer for 16 h. The protein solution was put on a CM-Sepharose Fast Flow (GE Healthcare) column (ϕ 1.6 × 10 cm) equilibrated with buffer F. After washing with buffer F, protease was eluted in the same buffer with a liner gradient of 0 to 200 mM NaCl. The protease fractions that were eluted as a single peak were pooled and precipitated with ammonium sulfate. The precipitate was dissolved in a small volume of buffer F and dialyzed against the same buffer for 16 h. The purified enzyme was confirmed to be homogeneous based on SDS-PAGE and staining with Coomassie brilliant blue R-250 of the gel.

Protein Measurement —— Protein concentration was determined spectrophotometrically using molar absorption coefficient constants (e_{280}) computed by ProtParam program (23). The concentrations of the mutated cucumisin propeptide were also determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories Inc., Hercules, CA) using wild-type cucumisin propeptide as a standard.

Assays for Protease and Protease Inhibitor, and Kinetic Measurements ——Cucumisin activity was assayed at pH 7.5 and 30°C using Glt-Ala-Ala-Pro-Leu-*p*NA as a substrate. The assay was started by adding 20 μl of cucumisin in 10 mM Na-phosphate buffer, pH 7.5, to 220 μl of substrate-propeptide mixture in assay buffer (150 mM Na-phosphate buffer, pH 7.5, containing 0.2 M NaCl). After incubating for 10 min at 30°C, the reaction was stopped by adding 240 μl of assay buffer containing 6.8 M guanidine hydrochloride. The mixture was centrifuged at 12,000 × *g* for 10 min at room temperature and the release of *p*-nitroaniline was measured by absorbance at 405 nm. All assays were performed in triplicate. K_m and K_i values were calculated by non-linear regression using GraphPad Prism 5.0 (GraphPad Software, Co. Ltd., La Jolla, CA). The K_i values were determined by curve fitting of the data using GraphPad Prism 5.0 to Equation 1.1 and Equation 1.2 (24).

$$V_i / V_{\max} = 1 - \frac{A - \sqrt{A^2 - 4[E][I]}}{2[E]} \quad \text{(Equ. 1.1)}$$

$$A = [E] + [I] + K_i\left(1 + \frac{[S]}{K_m}\right) \quad \text{(Equ. 1.2)}$$

In these equations, [E], [I], and [S] denote the enzyme, inhibitor, and substrate concentration, respectively. V_i and V_{max} mean the rate of *p*NA

release in the presence and absence of the inhibitor, respectively. Student's *t*-test was performed on experimental data (statistical significance $p < 0.05$).

Circular Dichroism (CD) Spectra Measurements —— CD spectra of the cucumisin propeptide and its mutants placed in a fused silica cuvette of 0.1 mm path length were recorded from 250 to 190 nm or from 250 to 210 nm in the presence of urea at 20°C using a JASCO J-720C spectrometer (JASCO Co. Ltd., Tokyo). Protein concentration was about 1 mg/ml, and the buffer used was 10 mM Na-phosphate buffer, pH 7.5, containing 0.2 M NaCl with or without urea. For measuring thermal denaturation, temperature was increased by 0.5°C/min from 20 to 90°C. Data were monitored for CD absorbance at 220 nm. For deconvolution, CD spectra were subjected to malticomponent secondary-structure analysis using the CONTIN/LL algorithm (25).

RESULTS

Sequence Alignments among Subtilases Propeptides —— The amino acid sequence of the cucumisin propeptide was compared with those of several plant subtilases and bacterial subtilisin E (Fig. 1). The cucumisin propeptide had 25-45% identities with those of the other plant subtilases. Although the sequences of plant subtilase propeptides had low identities (<20%) with that of subtilisin E, they contained conserved motifs N1 and N2 that were previously identified within bacterial subtilisins and appeared to be critical for protease domain folding (26). In the subtilisin E propeptide, hydrophobic residues located within N1 and N2 motifs (Val^{41}, Phe^{43}, Ile^{59}, Val^{66}, Leu^{80}, Val^{85}, Leu^{88}, Val^{94}, and Val^{97}) constituted a hydrophobic core. Among plant subtilase propeptides, most of these hydrophobic residues, including Val^{41}, Leu^{80}, Leu^{88}, Val^{94}, and Val^{97}, located within N1 and N2 motifs of subtilisin E were well conserved. It is assumed that these conserved hydrophobic amino acids are important for the functions of the cucumisin propeptide.

Most propeptides of bacterial subtilisins are known to be removed by autoprocessing. In case of plant subtilases, it was recently reported that the cleavage of the tomato SlSBT3 propeptide also occurred by autoprocessing (15). The NH_2-terminal residues of mature regions of plant subtilases, including SlSBT3 (Thr-Thr-Arg/His-Ser/Thr), are well conserved, suggesting that cleavages of most plant subtilase propeptides are also likely to occur autocatalytically, as with the SlSBT3 propeptide.

Expression and Purification of the Recombinant Cucumisin Propeptide ——The bacterial subtilisin propeptide plays an important role in inhibiting the active domain and folding of the mature enzyme (17-19). Because 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 similar functions as 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 so they were dissolved in a buffer containing 8 M urea and then purified by affinity column chromatography using Ni-Sepharose column, after which the urea was removed by dialysis as described in "Experimental Procedures". Since 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 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 the Recombinant Cucumisin Propeptide and Related Polypeptides ——Recombinant cuc-pro-WT strongly inhibited cucumisin (Fig. 3A). Pre-incubation 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 Glt-Ala-Ala-Pro-Leu-*p*NA as a substrate, the IC_{50} value for inhibition by cuc-pro-WT was approximately 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 tight-binding inhibitor of cucumisin. As shown in Fig. 3B, the IC_{50} values for inhibition of cucumisin by cuc-pro-WT increased linearly in proportion to the substrate concentration, which clearly demonstrated 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 if 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 K_i values of 62.0 ± 11 nM and 100 ± 12 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 previously reported (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 the Cucumisin Propeptide for the Inhibition of Mature Enzyme —— To evaluate the important region(s) within the cucumisin propeptide for the inhibition of mature enzyme, we expressed NH_2- or COOH-terminal truncated propeptides in *E. coli* as $(His)_6$-tagged proteins and measured the inhibitory activities of these recombinant propeptides against mature cucumisin (Fig. 4). The 9 NH_2-terminal amino acid-truncated propeptide (cuc-proΔN9) still had strong inhibitory activity against mature cucumisin (K_i = 14.7 ± 0.72 nM) comparable to that of cuc-pro (K_i = 7.1 ± 0.37 nM), while a 16 amino acid-truncated propeptide (cuc-proΔN16) showed a much weaker inhibition (K_i = 5.5 ± 0.73 μM). This suggested that the region from Asn^{32} to Met^{38} (NIYIVYM) was important for the inhibitory activity. Similarly, the 7 COOH-terminal amino acid-deleted propeptide (cuc-proΔC7) showed strong inhibition (K_i = 52.6 ± 6.5 nM) (*i.e.,* about 6 times less inhibition than cuc-pro), while the 14 COOH-terminal amino acid-deleted propeptide (cuc-proΔC14) resulted in no inhibition against cucumisin, suggesting that the region from Gly^{97} to Leu^{103} (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 Asn^{32} to Met^{38} and Gly^{97} to Leu^{103} 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 the Cucumisin Propeptide ——The hydrophobic amino acid residues Ile^{35}, Val^{36}, Tyr^{37}, Val^{98}, and Val^{101} in Asn^{32} to Met^{38} and Gly^{97} to Leu^{103} regions within the cucumisin propeptide are well conserved among plant subtilases (Fig. 1). Hydrophobic amino acids Val^{41}, Val^{94}, and Val^{97} in the subtilisin E propeptide, corresponding 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 in "Experimental Procedures." The inhibitory activities of these recombinant propeptide mutants were measured. In addition, Ile^{33} located within the Asn^{32} to 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 ± 0.79 nM), I35A (9.2 ± 0.68 nM) and Y37A (9.0 ± 0.85 nM) showed strong inhibitions comparable to cuc-pro. The mutant V36A, however, showed 5 times less inhibition (K_i = 37.4 ± 4.0 nM) than cuc-pro. These results indicate that Val^{36} is the most important amino acid in the Asn^{32} to Met^{38} 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} to 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 ± 0.56 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 ± 27 nM). However, another triple Ala-substitution mutant, V36A/V98A/V101A, showed only slightly weaker inhibition than V36A. These results suggest that Val^{98} and Val^{101}, which are conserved among many subtilases, are not too important for the inhibition and the possible cooperation between two hydrophobic regions.

The CD Spectroscopy for the 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 if 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 nm and 222 nm (Fig. 5A), corresponding to the CD spectrum of random coil and α-helix, respectively. β-Sheet also seems to contribute the spectrum around 215 nm. In contrast, the intensity around 215-230 nm remarkably decreased under denaturing conditions with 8 M 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% α-helix and 33.3 and 33.5% β-sheet, respectively (Table 3). These results indicate that both cucumisin propeptides, with or without 23 extra N-terminal amino acids, have secondary structures 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 N-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 $(His)_6$-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 approximately 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.

The CD Spectroscopy for Ala-substitution Mutants —— If the tripeptide $Ile^{35}Val^{36}Tyr^{37}$ 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 α-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 $Ile^{35}Val^{36}Tyr^{37}$ contributes 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 tight-binding competitive inhibitor of mature cucumisin. The K_i value of cuc-pro-WT was 6.2 ± 0.55 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 subtilisin. The amino acid sequences of ARA12 and RSP1 propeptides are about 36% identical to that of the cucumisin propeptide (Fig. 1) and the *Ki* 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 with 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 with the subtilisin BPN' propeptide (34). In spite of 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 active site clefts (28). However, it is unknown whether the cucumisin precursor can be activated by removing its propeptide by autoprocessing. Our finding that a cuc-proΔC7 propeptide mutant, without 7 COOH-terminal amino acids, still inhibited cucumisin with a K_i value of 52.6 ± 6.5 nM (Fig. 4), demonstrating that the 7 COOH-terminal amino acids in a propeptide are not essential for its inhibitory activity. Jean et al. also reported that a PfSUB-1 propeptide mutant without 11 COOH-terminal amino acids inhibited its cognate protease with about 14-fold larger K_i value (31). Regarding the processing of the prodomain of plant subtilase precursor, Cedzich *et al.* recently reported that the cleavage of the prodomain of tomato SlSBT3 occurs autocatalytically and zymogen maturation is an intramolecular process (15).

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 SlSBT3 in the endoplasmic reticulum (ER) is a prerequisite for passage through the secretory pathway using transient expression system in *N. 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 propeptide-enzyme complex, pH-regulated activation of furin in the secretory pathway and a pH sensor in the furin propeptide has been reported (36). To clarify 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 NH_2-teminal 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 NH_2-teminal 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 P_1 position and for Asp, Leu, and Ala at P_1' position (37), soybean C1 prefers Glu at P_1 and Glu/Gln at P_1' position (38), and tomato SlSBT3 shows a preference for Gln and Lys at P_1 and P_2 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, Asn^{32} to Met^{38} (NIYIVYM) and Gly^{97} to Leu^{103} (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), PfSUB-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 formed 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 due to the introduction of amino acid replacements, had lower K_i values of inhibition against subtilisin BPN' than 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 Tyr^{37} 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 were 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 that of cuc-pro (7.1 ± 0.37 nM), and I35A/V36A/Y37A inhibited cucumisin more weakly than 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. Since I35A had a strong inhibitory activity comparable to cuc-pro, the change of the secondary structure monitored around 203 nm for the Ala-substitution of Ile^{35}, 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%) and 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 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 was thought to be essential. The analyses of NH_2- 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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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Sequence alignment of the cucumisin propeptide with other subtilase propeptides. Propeptides with 4 NH_2-terminal residues of mature regions of 9 plant subtilases and subtilisin E are aligned using the ClustalW2 program (40). The numbers of amino acid residues begin from the first Met. The first amino acid residues of propeptides are predicted using the SignalP 3.0 program (41). Well-conserved amino acid residues within the propeptides (over 70% of the propeptides) and similar amino acid residues are shaded in black and gray, respectively. Gaps are denoted by dashes. Gray bars above the sequences represent the hydrophobic regions within the cucumisin propeptide. Two boxed regions show motifs N1 and N2. The secondary structures of the subtilisin E propeptide are shown below the sequences. The vertical arrow indicates the propeptide processing sites. ARA12 (14) and AIR3 (42) are from *Arabidopsis thaliana*. RSP1 (14) is from *Oryza sativa*. SBT1 (43) and P69A (44) are from *Solanum lycopersicum*. Protease C1 (45) is from *Glycine max*. AG12 (46) is from *Alnus glutinosa*. AF70 (47) is from *Picea jezoensis*. Subtilisin E (48) is from *Bacillus subtilis*.

Fig. 2. SDS-PAGE of purified propeptides. *A*, Cucumisin purified from melon fruit was subjected to 12.5% SDS-PAGE. *B-D,* Purified recombinant propeptides, cuc-pro-WT and cuc-pro (*B*), propeptides of ARA12, RSP1, and subtilisin E (*C*), and Ala-substitution mutants I33A, I35A, V36A, Y37A, I35A/V36A/Y37A, V98A, and V101 (*D*) were analyzed by SDS-PAGE (15% gel). M, molecular mass marker. Protein bands were stained with Coomassie brilliant blue R-250. Lane M, molecular mass marker.

Fig. 3. Inhibitory activities of plant subtilase propeptides against cucumisin. *A*, Inhibitory activity of the cucumisin propeptide against mature cucumisin. Purified recombinant cuc-pro-WT was incubated with cucumisin in 138 mM Na-phosphate buffer, pH 7.5, containing 0.2 M NaCl at 30°C for 10 min, and the remaining proteolytic activity of cucumisin was measured using Glt-Ala-Ala-Pro-Leu-*p*-NA as a substrate. *B*, Determination of the type of inhibition by the recombinant cucumisin propeptide. The IC_{50} values of cuc-pro-WT against cucumisin were plotted versus substrate concentrations. *C*, Inhibition curves of $(His)_6$-tagged recombinant propeptides of cucumisin (cuc-pro, circle), ARA12 (square), RSP1 (triangle), and subtilisin E (inverted triangle) against cucumisin. Data shown represents mean values obtained from three independent experiments and the error bars indicate S. E. of the mean.

Fig. 4. K_i values of the recombinant cucumisin propeptide and its mutants. The arrows show the lengths of the polypeptides. Ala-substituted residues are underlined. Inhibition assays were performed using Glt-Ala-Ala-Pro-Leu-*p*-NA as a substrate. Mean values ± S. E. of the mean of inhibition constant (K_i) are given in nM on the right for triplicate measurements. N.I. indicates no significant inhibition with 10 μM propeptide.

Fig. 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 depended on increasing (*B*) or decreasing (*C*) temperature. The Tm of cuc-pro is approximately 55°C. *D*, Structural changes of cuc-pro caused by denaturation with urea. Structural changes of cuc-pro monitored by negative ellipticity at 220 nm are depended on urea concentration. Data shown represents 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°C. Data shown represents mean values obtained from six independent experiments and the error bars indicate the S.E. of the mean.

Fig. 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).

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	GGGGGCTAGCAGATTGGATTCTGACGA
P-2	GGGGAAGCTTTCAATGAAGTTCGTTCATTT
P-3	GGGGGCTAGCAACATTTATATTGTAT
P-4	GGGGGCTAGCGGGAGGAAGCTAGAGGA
P-5	GGGGAAGCTTTCATAAAAACACAGACACCA
P-6	GGGGAAGCTTCACTCCATACTGGCAATCT
P-7	GGGGCCATGGCTTCTAGATTGGATTCTGA
P-8	GGGGGGATCCTCCTCCTCCGACCAAGGA
P-9	GGGGAAGCTTTCAGTGTAGCTCGTAA
P-10	GGGGGCTAGCTCACGCAAGCTGTACATA
P-11	GGGGAAGCTTTCACGCCGTCCTGTACCTC
P-12	GGGGGCTAGCGCCGGAAAAGCAGTACA
P-13	GGGGAAGCTTCAATATTCATGTGCAATATG

TABLE 2. Oligonucleotides used for cucumisin propeptide mutagenesis.
Mutated nucleotides were underlined.

Oligonucleotide sequence (5' to 3')	Mutants
GATGATGGAAAAAACGCTTATATTGTATACATGG	I33A
CCATGTATACAATATAAGCGTTTTTTCCATCATC	I33A
GAAAAAACATTTATGCGGTATACATGGG	I35A
CCCATGTATACCGCATAAATGTTTTTTC	I35A
CATTTATATTGCGTACATGGGGAGGAAG	V36A
CTTCCTCCCCATGTACGCAATATAAATG	V36A
CATTTATATTACCTACATGGGGAGGAAG	V36T
CTTCCTCCCCATGTAGGTAATATAAATG	V36T
CATTTATATTGTAGCGATGGGGAGGAAGCTAGAGG	Y37A
CCTCTAGCTTCCTCCCCATCGCTACAATATAAATG	Y37A
GAAAAAACATTTATGCGGCGGCCATGGGGAGGAAG	I35A/V36A/Y37A
CTTCCTCCCCATGGCCGCCGCATAAATGTTTTTTC	I35A/V36A/Y37A
CAGTATGGAGGGTGCGGTGTCTGTGTTTTTAAATG	V98A
CATTTAAAAACACAGACACCGCACCCTCCATACTG	V98A
GAGGGTGTGGTGTCTGCGTTTTTAAATGAAATG	V101A
CATTTCATTTAAAAACGCAGACACCACACCCTC	V101A
GAGGGTGCGGTGTCTGCGTTTTTAAATGAAATG	V98A/V101A
CATTTCATTTAAAAACGCAGACACCGCACCCTC	V98A/V101A

TABLE 3. Secondary-structure content of cuc-pro and four Ala-substitution mutants.

Secondary-structure contents were calculated using CONTIN/LL algorism (25).

Propeptides	α-helix (%)	β-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	33.1
I35A/V36A/Y37A	9.3	33.0