Molecular Basis for Auto- and Hetero-catalytic Maturation of a Thermostable Subtilase from Thermophilic Bacillus sp. WF146*

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Background: The autocatalytic maturation mechanisms of subtilases are well known, but little is known about their hetero-catalytic maturation.

Results: The N-terminal propeptide of the WF146 protease can be removed via both cis- and trans-processing reaction-initiated maturation pathways.

Conclusion: The WF146 protease is intrinsically able to mature either autocatalytically or hetero-catalytically.

Significance: Our work provides new insights into maturation mechanisms of subtilases.

The proform of the WF146 protease, an extracellular subtilase produced by thermophilic Bacillus sp. WF146, matures efficiently at high temperatures. Here we report that the proform, which contains an N-terminal propeptide composed of a core domain (N*) and a linker peptide, is intrinsically able to mature via multiple pathways. One autocatalytic pathway is initiated by cis-processing of N* to generate an autoprocessed complex N*-IWT, and this step is followed by truncation of the linker peptide and degradation of N*. Another autocatalytic pathway is initiated by trans-processing of the linker peptide followed by degradation of N*. Unlike most reported subtilases, the maturation of the WF146 protease occurs not only autocatalytically but also hetero-catalytically whereby heterogeneous proteases accelerate the maturation of the WF146 protease via trans-processing of the proform and N*-IWT. Although N* acts as an intramolecular chaperone and an inhibitor of the mature enzyme, the linker peptide is susceptible to proteolysis, allowing the trans-processing reaction to occur auto- and hetero-catalytically. These studies also demonstrate that the WF146 protease undergoes subtle structural adjustments during the maturation process and that the binding of Ca²⁺ is required for routing the proform to mature properly at high temperatures. Interestingly, under Ca²⁺-free conditions, the proform is cis-processed into a unique propeptide-intermediate complex (N*-I²) capable of re-synthesis of the proform. Based on the basic catalytic principle of serine proteases and these experimental results, a mechanism for the cis-processing/re-synthesis equilibrium of the proform and the role of the linker peptide in regulation of this equilibrium has been proposed.

Subtilisin-like serine proteases (subtilases) are widely distributed in bacteria, archaea, and eukaryotes and play important roles in a variety of biological processes, including nutrition, protein catabolism, and zymogen activation (1, 2). Extracellular subtilases are generally produced as inactive precursors composed of a signal peptide, an N-terminal propeptide, and a mature domain. The inactive precursors undergo maturation to release the enzymatic activity, and a stepwise maturation process enables precise regulation of the activity both spatially and temporally (3). The signal peptide mediates the secretion of the enzyme across the cytoplasmic membrane, where it is cleaved by a signal peptidase. The N-terminal propeptide usually functions as an intramolecular chaperone to facilitate the correct folding of the mature domain and also as a potential inhibitor of its cognate mature enzyme to prevent premature protease activation, thereby maximizing the maturation efficiency (4). Thermophilic subtilases have attracted considerable attention because of their contributions to the understanding of temperature adaptation mechanism of enzymes as well as their industrial potential (2, 5, 6). Investigation into the maturation processes of subtilases adapted to different temperatures not only helps us to further understand their temperature adaptation strategies but also provides valuable information for preparation of active subtilases of industrial potential.

The maturation processes of some subtilases have been extensively characterized and found to proceed autocatalytically (4); however, the maturation process varies greatly between different subtilases in terms of the structural change that occurs during maturation, the metal ion dependence, etc. For instance, with the aid of the N-terminal propeptide, pro-subtilisin E from Bacillus subtilis folds into a partially structured folding intermediate and then undergoes structural changes to yield a native-like folded proform. Subsequently, the proform converts into a non-covalently associated propeptide-subtilisin complex via intramolecular autoprocessing of the N-terminal propeptide. Finally, the release and degradation of the N-terminal propeptide from the autoprocessed complex result in an active protease (4). Like subtilisin E, hyperthermophilic Tk-subtilisin from Thermococcus kodakaraensis matures from its proform upon autoprocessing and degradation of the propeptide; however, the maturation of Tk-subtilisin differs from that of subtilisin E in that maturation is Ca²⁺-dependent and the enzyme structure is not seriously changed during maturation (7, 8).
Although the autocatalytic maturation mechanisms of some subtilases have been well established, subtilases have also been shown to mature in a hetero-catalytic manner. A previous study of *Bacillus amylo liquefaciens* subtilisin BPN’ revealed that its active-site mutant precursor remained associated with the membrane after expression in *B. subtilis* but could be processed to a mature form and released into the medium by active subtilisin from *B. subtilis* and *Bacillus licheniformis* (9). Our study on a subtilase (protease CDF) from *Thermoactinomyces* sp. CDF revealed that an active-site mutant proform was processed to the mature form by proteinase K in vitro (10). Recently, the AprV5 subtilase from *Dichelobacter nodosus* was reported to be responsible not only for its own maturation but also for optimal processing of two other subtilases (ApV2 and BprV) from the same strain (11). Compared with the known mechanisms of autocalytic maturation, the molecular basis of hetero-catalytic maturation of subtilases remains to be elucidated.

The WF146 protease is a thermostable subtilase secreted by thermophilic *Bacillus* sp. WF146, and its precursor is composed of a signal peptide, an N-terminal propeptide, and a mature domain (12, 13). The recombinant proform of the wild-type WF146 protease matures effectively at high temperatures (e.g. 60 °C), but an active-site mutant proform does not, suggesting that maturation proceeds autocatalytically (14). In a subsequent study, we found that the N-terminal propeptide of an active-site mutant proform was more susceptible to limited proteolysis by active WF146 protease or trypsin than the mature domain of the mutant proform (15). These findings lead us to postulate that the maturation of the WF146 protease can occur hetero-catalytically by trans-processing of the N-terminal propeptide. Therefore, to address this hypothesis, we investigated the auto- and hetero-catalytic maturation processes of the WF146 protease in detail. In the autocalytic maturation pathway, the proform converted to the mature enzyme via an intermediate by stepwise autoprocessing of the N-terminal propeptide concomitant with subtle structural adjustments. Meanwhile, the proform could be trans-processed to the mature form either by active WF146 protease or by heterogeneous proteases, confirming the existence of a hetero-catalytic maturation pathway. Interestingly, a unique propeptide-intermediate complex that formed under Ca²⁺-free conditions was capable of re-synthesis of the proform. Based on these results, a hypothetical model of the maturation process of WF146 protease was proposed, and a mechanism for cis-processing/re-synthesis of the proform was suggested.

**TABLE 1**

| Primer | Oligonucleotide sequence* |
|--------|---------------------------|
| P-F    | 5'-AAATGCAGCCATGGATAGGCGAAAGCACAAGTTGCG-3' |
| P-R    | 5'-GCTCTCAGTGGTGCTGGCGGCTGGATGAGCA-3' |
| ISC-F  | 5'-CATCAGGCGACGGCGATGCG-3' |
| MAT-F  | 5'-AACATATGGCGACTTCCCTGATGGAAC-3' |
| PRO-F  | 5'-TTGTTAATAGCATGACGGACCCACCCACCCACACGGTGGATGAGCA-3' |
| GAGGACAAAGATG-3' |
| PRO-R  | 5'-GTTGTCACTCAAAAGCTGGATGCGCCCTCTG-3' |
| PROC-R | 5'-GTTGTCACTCAACGGCTGACGCGTGTTTGACCA-3' |

* Underlined sequences indicate restriction enzyme sites. The mutated nucleotide is boxed. Italicized sections indicate the His$_{12}$-tag encoding DNA sequences.

**TABLE 2**

| Expression plasmid | Primer pair | Restriction enzyme site |
|--------------------|-------------|-------------------------|
| pS249C             | ISC-F/P-R (megaprimer) | Mscl-Xhol             |
| pMAT               | MAT-F/P-R | Ndel-Xhol              |
| pPRO85             | PRO-F/PRO-R | Ndel-Xhol              |
| pPRO73             | PRO-F/PROC-R | Ndel-Xhol              |

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes, T4 DNA ligase, and Pfu DNA polymerase were purchased from Fermentas (Burlington, Canada). PMSF, azocasein, succ-AAPF-pNA, proteinase K, and subtilisin A were purchased from Sigma.

**Strains and Growth Conditions**—*Escherichia coli* DH5α and *E. coli* BL21 (DE3) were used as the hosts for cloning and expression, respectively. Bacteria were grown at 37 °C in Luria-Bertani medium supplemented with kanamycin (30 μg/ml) as needed.

**Plasmid Construction and Mutagenesis**—We have previously constructed the expression plasmid pHSNM for the proform of wild-type WF146 protease (pro-WT). A pelB signal peptide was fused at the N terminus to direct the proform via a disulfide bond between Cys$^{52}$ and Cys$^{65}$ to the *E. coli* periplasm for proper disulfide bond formation and enzyme folding (13). The megaprimer PCR method (16) was used to construct the expression plasmid pS249A, which contains the gene encoding the proform of WF146 protease with the Ser$^{249}$ to Ala mutation (pro-S249A) (14). Here, we used a mutagenic primer (ISC-F) to construct an expression plasmid pS249C for the proform of WF146 protease with the Ser$^{249}$ to Cys mutation (pro-S249C; Tables 1 and 2). To construct the expression plasmids for the N-terminal propeptide deletion mutant of WF146 protease (Mat-WT) and the N-terminal propeptide (Pro85 and its core domain (Pro73), the genes encoding these polypeptides were amplified by PCR from pHSNM with the primer pairs listed in Table 2 and were inserted into pET26b to generate the plasmids pMAT, pPRO85, and pPRO73, respectively. All recombinant plasmids were confirmed by DNA sequencing.

**Expression and Purification**—Expression of the recombinant proteins in *E. coli* BL21(DE3) was carried out as described previously (12). Then the cells were harvested and suspended in a Ca²⁺-containing buffer (50 mM Tris-HCl, 10 mM CaCl$_2$, 10 mM NaCl, pH 8.0) followed by sonication on ice. Soluble and insoluble fractions were separated by centrifugation at 13,400 × g for 10 min (4 °C). Although Mat-WT formed inclusion bodies.
Maturation of Thermostable WF146 Protease

the other recombinant proteins were expressed in soluble form. The soluble fraction containing the recombinant proteins were subjected to affinity chromatography on a Ni²⁺-charged Chelating Sepharose™ Fast Flow resin (Amersham Biosciences) column (1.6 × 20 cm) equilibrated with buffer A (50 mM Tris-HCl, 0.5 mM NaCl, pH 7.5) containing 9 mM imidazole. After washing the column with buffer A containing 40 mM imidazole, the bound His-tagged proteins were eluted with buffer A containing 300 mM imidazole and dialyzed against the Ca²⁺-containing buffer at 4 °C. To prepare the sample for investigation of the processing of pro-WT under Ca²⁺-free conditions, the eluted fraction containing the proform was dialyzed against a Ca²⁺-free buffer (50 mM Tris-HCl, 10 mM NaCl, 2 mM EDTA, pH 8.0) at 4 °C.

For purification of mature WF146 protease, pro-WT was incubated in the Ca²⁺-containing buffer at 60 °C for 1 h to activate the enzyme and then subjected to affinity chromatography on a bacitracin-Sepharose™ 4B (Amersham Biosciences) column (1.6 × 20 cm) equilibrated with buffer B (50 mM Tris-HCl, 0.5 mM NaCl, pH 8.0). After washing with buffer B, the enzyme was eluted with the same buffer containing 25% isopropanol alcohol. Finally, the eluted fraction containing the purified enzyme was dialyzed against the Ca²⁺-containing buffer overnight at 4 °C.

The enzyme solution was concentrated with a Micron YM-3 centrifugal filter (Millipore, Bedford, MA) as needed. The protein concentrations of the purified enzyme samples were measured using the Bradford method (17) with BSA as the standard.

Renaturation and Activation of Mat-WT—The inclusion bodies of Mat-WT were isolated as described by Tang et al. (18) and solubilized in 50 mM Tris-HCl buffer, pH 7.5, containing 8 mM urea and 10 mM DTT at a protein concentration of 50 μg/ml. Subsequently, an equal molar concentration of Pro73 or Pro85 was added to the solution, and the mixture was incubated at 30 °C for 1 h. Thereafter, the solution was dialyzed against the Ca²⁺-containing buffer at 4 °C overnight and then subjected to heat treatment at 60 °C for 2 h to activate the enzyme. The generated mature enzymes were purified by affinity chromatography on a bacitracin-Sepharose™ 4B column as mentioned above.

Enzyme Activity Assay—The azocaseinolytic activity of the enzyme was determined at 60 °C for 15 min in 400 μl of reaction mixture containing 0.25% (w/v) azocasein and 200 μl of enzyme sample in the Ca²⁺-containing buffer. The reaction was terminated by the addition of 400 μl of 40% TCA to the reaction mixture. After incubation at room temperature for 15 min, the mixture was centrifuged at 13,400 × g for 10 min, and the absorbance at 335 nm (A₃₃₅) of the supernatant was measured in a 1-cm cell. One unit of activity was defined as the amount of enzyme required to increase the A₃₃₅ by 0.01 unit/min under the conditions described above.

The activity of enzyme toward suc-AAPF-pNA (0.2 mM) was measured at 60 °C in the Ca²⁺-containing buffer. The activity was recorded by monitoring the initial velocity of hydrolysis of suc-AAPF-pNA at 410 nm in a thermostatted spectrophotometer (Cintra 10e, GBC, Australia), calculated on the basis of an extinction coefficient for p-nitroaniline (pNA) of 8480 M⁻¹cm⁻¹ at 410 nm. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of pNA/min under assay conditions. Using suc-AAPF-pNA as the substrate, kinetic constants for enzymes were determined as described previously (14).

SDS-PAGE and Immunoblot Analysis—SDS-PAGE was carried out with the glycine-Tris (19) or Tricine-Tris buffer system (20). To prevent self-degradation of the protease during sample preparation (boiling) or electrophoresis, the protease was precipitated by TCA at a final concentration of 20%. After incubation at room temperature for 15 min, the precipitated proteins were recovered by centrifugation at 13,400 × g for 10 min and then resuspended in ice-cold acetone. Thereafter, the proteins were recovered by centrifugation at 13,400 × g for 10 min, air dried, and then subjected to SDS-PAGE. The levels of the target proteins were estimated from the band intensities on the SDS-PAGE gels using GeneTools gel imaging software (Syngene, Cambridge, UK). The His-tag monoclonal antibody (Novagen, Darmstadt, Germany) was used for immunoblot analysis as described previously (10).

N-terminal Amino Acid Sequencing—After separation by SDS-PAGE, the proteins were electroblotted to a PVDF membrane and then stained with Coomassie Brilliant Blue R-250. The target protein bands were excised and subjected to N-terminal amino acid sequence analysis using a Proces 492 cLC peptide sequencer (Applied Biosystems).

CD Spectroscopy—The far-UV (200–260 nm) CD spectra of protein samples (0.1 mg/ml) were measured using a Jasco J-810 spectropolarimeter (Jasco Corp., Tokyo, Japan) in a cell with an optical path length of 1 mm. The mean residue ellipticity [θ] (deg·cm²·dmol⁻¹) was calculated with an average amino acid molecular mass of 104.3 (the proform and the autoprocessed complex) or 102.1 (the mature form). The spectra were recorded in the Ca²⁺-containing buffer or in the Ca²⁺-free buffer as indicated. Before being subjected to CD analysis, the mature WF146 protease was inactivated by 10 mM PMSF at 30 °C for 30 min and then dialyzed against the Ca²⁺-containing or the Ca²⁺-free buffer at 4 °C overnight.

RESULTS

The Autocatalytic Maturation of WF146 Protease Involves Stepwise Processing of the N-terminal Propeptide—To investigate the autocatalytic maturation process of the WF146 protease, the purified pro-WT (Fig. 1A) was incubated at 60 °C, and the processed products were resolved by SDS-PAGE and iden-
incubated for 5 min exhibited a slightly faster migration rate than the pro-form of the enzyme with an N-terminal sequence identified as ATPS (Fig. 1B). Meanwhile, the appearance of proforms derived from N* through proteolytic cleavage of the Thr78 peptide bond by active enzyme that matures at the early stages of the maturation process. Furthermore, additional processed products were also detected (Fig. 1B). The 25-kDa fragment (FC) has been previously identified as an autoprotoolytic product of the WF146 protease (13). The N* fragment (band d) of which the first four amino acids identical to those of band b (DESK and MDES, Fig. 1C), representing the processed N-terminal propeptide. The smallest N2 fragment (band e), of which the first four amino acids were identified as SSDK (Fig. 1C), is most likely derived from N* through proteolytic cleavage of the Thr77-Ser78 peptide bond by the mature WF146 protease.

A minor species with the N-terminal sequence EEFE migrated between the bands of pro-WT and the mature enzyme (band e, Fig. 1B and C). In addition, this minor species was detected by immunoblotting using a monoclonal antibody against the His tag (data not shown), which was fused to the C terminus of pro-WT. Therefore, it may represent a maturation intermediate (1WT) derived from pro-WT via autoprocessing of its N-terminal propeptide at Ala13-Glu12 (Fig. 1C). To test this possibility, we utilized active-site mutants or the serine protease inhibitor PMSF to block various steps in the maturation pathway to identify the possible maturation intermediates. The catalytic Ser249 residue of the WF146 protease has been confirmed to mediate autoprocessing of the N-terminal propeptide (14), as evidenced by the fact that the active-site mutant pro-S249A remained unprocessed after 3 h of incubation at 60 °C (Fig. 2A). It is known that substitution of the active-site Ser with Cys greatly reduces enzymatic activity of subtilisins (e.g. subtilisin E and Tk-subtilisin) but does not eliminate their autoprocessing activities, such that the maturation process is blocked subsequent to the first proteolytic cleavage of the N-terminal propeptide, which facilitates the isolation of sta-

**TABLE 3**

| Proteins                     | Predicted a | Apparent b |
|------------------------------|-------------|------------|
| Pro-WT, Pro-S249A, or Pro-S249C | 42.4 ± 43 | 8.1 ± 9    |
| Pro85                        | 10.5 ± 12   | 7.4 ± 7    |
| Pro73                        | 9.1 ± 10    | 8.1 ± 9    |
| Mat-WT                       | 33.1 ± 35   | 34.4 ± 36  |
| Pro73/His                     | 31.9 ± 34   | 34.4 ± 36  |
| IWT/WT, I249S/C              | 33.1 ± 34   | 34.4 ± 36  |
| N*                           | 8.1 ± 9     | 8.1 ± 9    |

*Estimated by Tricine-SDS-PAGE analysis.
*These proteins have a fused His tag.

![Figure 1](https://example.com/figure1.png)

*FIGURE 1. Autocatalytic maturation process of pro-WT. A, schematic representation of the primary structures of pro-WT and its derivatives. The locations of the active-site residues and the N- and C-terminal residues of each region are shown. The fused pelB signal peptide (Pel), the N-terminal propeptide (N), and its core domain (N*), the mature domain (M), and the fused His tag (H) are indicated. B, SDS-PAGE analysis of the maturation process. Purified pro-WT (0.5 µg) was incubated at 60 °C in the Ca2+-containing buffer. At the time intervals indicated, aliquots were withdrawn and subjected to Tricine-SDS-PAGE. The positions of the proform (P), the intermediate (IWT), the mature form (M), the autoproteolytic product (FC), and the fragments derived from the N-terminal propeptide (N*) and N2 are indicated. The bands a–f were subjected to N-terminal sequencing. C, N-terminal amino acid sequences of the processed products. The first four identified residues of the protein bands are indicated in Figs. 1A (a–f), 2A (g), and 68 (h–j) are underlined. The sequences of the fused pelB signal peptide, the core domain of the N-terminal propeptide (N*), the linker peptide, and the N-terminal part of the mature domain are shown. The numbers represent the positions of the amino acid residues starting from the N terminus of the mature domain.*

of the mature enzyme at early stages (0–5 min, Fig. 1B). N-terminal sequencing of the proform band that was incubated for 5 min (band b, Fig. 1B) also revealed two classes of polypeptides, and one possesses an identical N-terminal sequence (MDES) to that of the polypeptide lacking the pelB signal peptide detected in band a (Fig. 1C). The N-terminal sequence of the other polypeptide in band b was identified as DESK, which corresponds to the sequence that follows Met85 immediately downstream of the pelB signal peptide (Fig. 1C). This finding implies that the fused pelB signal peptide of the band a polypeptide could be cleaved at the Met85-Asp84 bond by active enzyme that matures at the early stages of the maturation process. Therefore, it may represent a maturation intermediate (1WT) derived from pro-WT via autoprocessing of its N-terminal propeptide at Ala13-Glu12 (Fig. 1C). To test this possibility, we utilized active-site mutants or the serine protease inhibitor PMSF to block various steps in the maturation pathway to identify the possible maturation intermediates. The catalytic Ser249 residue of the WF146 protease has been confirmed to mediate autoprocessing of the N-terminal propeptide (14), as evidenced by the fact that the active-site mutant pro-S249A remained unprocessed after 3 h of incubation at 60 °C (Fig. 2A). It is known that substitution of the active-site Ser with Cys greatly reduces enzymatic activity of subtilisins (e.g. subtilisin E and Tk-subtilisin) but does not eliminate their autoprocessing activities, such that the maturation process is blocked subsequent to the first proteolytic cleavage of the N-terminal propeptide, which facilitates the isolation of sta-

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Maturation of Thermostable WF146 Protease

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The Proform Can Be Trans-processed into Its Mature Form by Active WF146 Protease—Although cis-processing of N* was independent of protein concentration (Fig. 2C), the maturation of the WF146 protease was accelerated at higher protein concentrations (Fig. 4A). Because the maturation process involved not only cis-processing of N* to generate I\(^{WT}\) but also trans-processing of I\(^{WT}\) to generate the mature form, one reasonable explanation for the protein concentration-dependent maturation is that trans-processing of I\(^{WT}\) to the mature form is accel-

\[\text{N*-IS249C, IWT and N* formed an autoprocessed complex (N*) that was co-eluted from the Ni\(^2+\)-charged column (Fig. 1C).} \]

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erated with increased protein concentration. Furthermore, the result of band intensity quantification revealed that less than half of the pro-WT molecules were cis-processed to IWT after incubation at 60 °C for 0.5 h in the presence of PMSF (Fig. 2C). If the cis-processing of the proform to IWT is an essential step in the maturation of the WF146 protease, most of the pro-WT molecules would remain intact, and a small amount of the mature forms would be generated via IWT within 0.5 h in the absence of PMSF; however, at the same protein concentration and in the absence of PMSF, almost all of the pro-WT molecules had converted to mature forms within 20 min (Fig. 4A),

**TABLE 4**

| Activity and stability parameters of mature WF146 protease | Kinetic constants | $t_{1/2}$ (80 °C) |
|------------------------------------------------------------|------------------|-----------------|
| **Enzymes** | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | 0.1 mM CaCl$_2$ | 5 mM CaCl$_2$ | 10 mM CaCl$_2$ |
| A | 0.61 ± 0.04$^d$ | 348 ± 27$^d$ | < 10 | 32 | 60$^d$ |
| B | 0.68 ± 0.07 | 310 ± 17 | < 10 | 34 | 56 |
| C | 0.70 ± 0.02 | 329 ± 12 | < 10 | 30 | 58 |

$^a$ The mature WF146 protease was derived either from pro-WT (A) or from Mat-WT that was renatured in the presence of Pro73 (B) or Pro85 (C) as described under "Experimental Procedures.

$^d$ The kinetic constants were determined at 60 °C using suc-AAPF-pNA as the substrate as described under "Experimental Procedures." The values are expressed as mean ± S.D. of three independent experiments.

$^e$ The enzymes (0.1 μM) in 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM NaCl and the indicated concentrations of CaCl$_2$ were incubated at 80 °C. Residual activity was measured at appropriate time intervals using suc-AAPF-pNA (0.2 mM) as the substrate in 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM NaCl and 10 mM CaCl$_2$. The values are expressed as the means of three independent experiments, and the S.W. are less than 10%.

$^d$ Data were from Yang et al. (14).
suggesting that trans-processing of the proform directly into the mature form had occurred during the maturation process. Supporting evidence came from the finding that pro-S249A was also trans-processed to the mature form by active WF146 protease (Fig. 4B). In addition, the size of the trans-processed N-terminal propeptide processed from pro-S249A by active WF146 protease (N*, Fig. 4B) was similar to that of the processed N* detected during the autocatalytic enzyme maturation process (Fig. 1A), indicating that the initial trans-processing site is located in the linker peptide of the proform.

To investigate whether the mature form generated via trans-processing of the proform is active, pro-WT (0.05 μM) was incubated at 60 °C alone or with supplemental active WF146 protease (Fig. 4A). After 40 min, ~40% of the pro-WT molecules that were incubated alone remained intact. In contrast, when incubated with supplemental active WF146 protease, all pro-WT molecules were converted into mature forms, and the activity of the newly generated mature forms was ~37% higher than that of the enzyme incubated without supplemental WF146 protease (Fig. 4A). This result indicates that the proform of the WF146 protease could be directly trans-processed to the active mature form.

**Heterogeneous Proteases Accelerate the Maturation of WF146 Protease by Trans-processing of the Proform and the Autoprocessed Complex**—To investigate whether the maturation of WF146 protease can proceed hetero-catalytically, the proform (pro-S249A) and the autoprocessed complex (N*-I5249C) were incubated at 60 °C with proteinase K or subtilisin A. As shown in Fig. 5A, both pro-S249A and N*-I5249C were converted into the mature form in the presence of proteinase K or subtilisin A, implying that the proform and the autoprocessed complex of WF146 protease can be trans-processed to the mature form by heterogeneous proteases. Notably, the fragments of the N-terminal propeptide processed from pro-S249A by proteinase K and subtilisin A were similar to that processed by active mature WF146 protease (Figs. 4B and 5A), indicating that the linker peptide of the proform is not only susceptible to WF146 protease but also to heterogeneous proteases.

We next investigated whether heterogeneous proteases could accelerate WF146 protease maturation and whether the generated mature form is active. In the presence of different concentrations of proteinase K, pro-WT (0.5 μM) had converted into mature forms within 10 min at 60 °C, whereas more than half pro-WT molecules remained as proforms after a 10-min incubation in the absence of proteinase K (Fig. 5B, left panel). It was noticed that the mature form could be detected in the initial mixed sample (lane 0 min), and its level increased as the proteinase K concentration enhanced (Fig. 5B, left panel), implying that the conversion of pro-WT to the mature form had occurred immediately after the addition of proteinase K. Meanwhile, the presence of proteinase K also accelerated WF146 protease maturation at 25 °C (Fig. 5C). These results indicate that WF146 protease maturation was accelerated with the aid of proteinase K. It was found that, after incubation for 40 min, the levels of activity released by the WF146 protease, activated either in the absence or presence of lower concentrations of proteinase K (0.35 μM), were similar to each other (Fig. 5B, right panel). Moreover, in both cases the activity levels and the band patterns of the activated WF146 protease remained almost unchanged as the incubation period was extended to 240 min (Fig. 5B), indicating that the mature forms generated under both conditions were fully activated. However, the activ-
ity level of activated WF146 protease tended to decrease with the increase of proteinase K concentrations (0.6 and 1.2 mM), but an extended incubation period (240 min) did not lead to a further loss of the activity (Fig. 5B, right panel). One reasonable explanation for this is that, besides the linker peptide, another region(s) of the pro-WT molecule may have also suffered proteolysis under highly proteolytic environments, leading to the degradation of the proform and a lower yield of active mature form; however, the WF146 protease, once activated, would become resistant to proteolysis, most likely due to its structural adjustment during the maturation process. Consistent with our previous finding that WF146 protease showed no loss of activity after incubation at 60 °C for more than 1 h (12), the mature forms that were activated in the presence of proteinase K remained stable throughout a 200-min incubation at 60 °C (40–240 min, Fig. 5B), indicating that they had achieved high thermostability. Taken together, these results suggest that the maturation of WF146 protease can, indeed, occur hetero-catalytically. Nevertheless, the acceleration of WF146 protease maturation in the presence of high concentrations of heterogeneous proteases may be accompanied by a reduction of the level of the activated mature enzyme.

Under Ca\textsuperscript{2+}-free Conditions, the Proform Is Cis-processed into a Unique Propeptide-Intermediate Complex Capable of Re-synthesis of the Proform—Ca\textsuperscript{2+} contributes thermostability to the WF146 protease (13). When pro-WT was incubated at 60 °C for 1 h with different CaCl\textsubscript{2} concentrations, the yield of mature enzyme increased as the CaCl\textsubscript{2} concentration increased from 0.01 to 1 mM, although the yield remained unchanged at the CaCl\textsubscript{2} concentrations from 1 to 10 mM (Fig. 6A). This result indicates that the proper maturation of WF146 protease is Ca\textsuperscript{2+}-dependent and that the low maturation efficiency at lower CaCl\textsubscript{2} concentrations (e.g. 0.01–0.1 mM) is most likely due to the destabilization and autoproteolysis of the proform and the mature form under Ca\textsuperscript{2+}-limiting conditions.

In the Ca\textsuperscript{2+}-free buffer that contains 2 mM EDTA, pro-WT was not converted into the mature form after incubation at 60 °C for 1 h but was processed into three fragments (Fig. 6B, lanes S1 and S2) that showed no proteolytic activity. This result remained unchanged even after extension of the incubation period up to 4 h (data not shown). This finding indicates that the binding of Ca\textsuperscript{2+} is required for routing the proform to complete the maturation process. The fragment I\textsuperscript{E} (Fig. 6B, band i) has an N terminus that is identical to that of I\textsuperscript{WT} and I\textsuperscript{S2-49C} (EFEE, Fig. 2C), and this fragment represents the intermediate generated under Ca\textsuperscript{2+}-free conditions by cis-processing of N* at the bond Ala\textsuperscript{13}–Glu\textsuperscript{12}. The four N-terminal amino acid residues of the two small fragments (Fig. 6B, bands i and j) were identified as MKYL and MDES, representing the processed N* form with and without the fused peB signal peptide, respectively (Fig. 2C). Meanwhile, N* was co-eluted with I\textsuperscript{E} from the Ni\textsuperscript{2+}-charged column (data not shown), suggesting that these species formed an autoprocessed complex (N*-I\textsuperscript{E}).

Interestingly, a temperature shift from 60 to 25 °C resulted in a decrease in the amounts of both I\textsuperscript{E} and N* accompanied by an increase in the amount of the proform (Fig. 6B, lane S3). This result implies that the Ala\textsuperscript{13}–Glu\textsuperscript{12} bond was re-ligated and that N*-I\textsuperscript{E} was capable of re-synthesis of the proform. As the incubation temperature was subsequently shifted from 25 to 60 °C, the re-synthesized proform was again processed into N*–I\textsuperscript{E} (Fig. 6B, lane S4). The reversible conversion between the proform and N*–I\textsuperscript{E} was induced not only by a temperature shift but also by changes in Ca\textsuperscript{2+} concentrations. More specifically, an excess of Ca\textsuperscript{2+} led to the re-synthesis of the proform, whereas an excess of EDTA resulted in the formation of N*–I\textsuperscript{E} (Fig. 6B, lanes S5 and S6). When incubated at 60 °C with an excess of Ca\textsuperscript{2+}, the re-synthesized proform was converted into the mature form (Fig. 6B, lanes S7 and S8), indicating that this enzyme adopts a maturation–competent state upon binding Ca\textsuperscript{2+}. In addition, the inhibitor PMSF was unable to prevent the reversible conversion between the proform and N*–I\textsuperscript{E} (Fig. 6C), suggesting that the active sites were shielded from solvent and that the cleavage/re-ligation reactions occurred intramolecularly.

As shown in Fig. 2C, N*-I\textsuperscript{WT} that formed in the presence of Ca\textsuperscript{2+} could be processed to the mature form. To determine
whether the autoprocessed complex could be re-synthesized to the proform, N*-IWT was generated by incubating pro-WT at 60 °C in the presence of Ca\(^{2+}\) and PMSF followed by treatment with an excess of EDTA. The EDTA-treated N*-IWT was incapable of re-synthesis of the proform after a temperature shift from 60 to 25 °C (Fig. 6D, lanes S2‘ and S3‘). In addition, the EDTA-treated N*-IWT complex was converted to the mature form by incubation at 60 °C in the presence of excess Ca\(^{2+}\) (Fig. 6D, lanes S2‘ and S5‘), in contrast to the re-synthesis of the proform that occurred in the case of N*-IE (Fig. 6B, lanes S2 and S5). This finding indicates that N*-IWT is distinct from N*-IE and represents a unique propeptide-intermediate complex that forms under Ca\(^{2+}\)-free conditions.

The WF146 Protease Undergoes Subtle Structural Adjustments during the Maturation Process—To examine the structural changes that occur during the maturation process, the far-UV CD spectra of pro-S249A, N*-IS249C, and mature WF146 protease, which represent the proform, the autoprocessed complex, and the mature form, respectively, were measured. In Ca\(^{2+}\)-containing buffer, the proform and the autoprocessed complex displayed similar but not identical spectra at 60 °C, wherein the latter exhibited slightly lower mean residue ellipticity values than the former in the wavelength range 208–230 nm (Fig. 7A). This result implies that cis-processing of the N-terminal propeptide leads to subtle structural changes. The structural difference between the two forms became more evident under Ca\(^{2+}\)-free conditions, as evidenced by the significantly higher mean residue ellipticity values of the autoprocessed complex than the values of the proform at 60 °C (Fig. 7B). Additionally, in contrast to their spectra with a double minimum around 210 and 220 nm in Ca\(^{2+}\)-containing buffer, the spectra of the two forms at 60 °C showed a single minimum around 210 nm in Ca\(^{2+}\)-free buffer (Fig. 7, A and B), demonstrating the importance of Ca\(^{2+}\) binding in maintaining the native-like structures of the proform and the autoprocessed complex. Meanwhile, the involvement of Ca\(^{2+}\) binding in stabilization of the two forms was further supported by the fact that the proform and the autoprocessed complex were fully degraded by proteinase K under Ca\(^{2+}\)-free conditions (data not shown) as opposed to being trans-processed to the mature form by the same enzyme in Ca\(^{2+}\)-containing buffer (Fig. 5A).

In the presence of Ca\(^{2+}\) and at 60 °C, the mature form displayed mean residue ellipticity values that were almost identical to those of the autoprocessed complex at wavelengths above 210 nm; however, the mature form exhibited lower values than the autoprocessed complex in the wavelength range of 200–210 nm (Fig. 7A), likely due to the lack of the N-terminal propeptide. At 25 °C, all of the proform, the autoprocessed complex, and the mature form showed a similar decrease in mean residue ellipticity values around 220 nm in the presence of CaCl\(_2\) compared with their spectra at 60 °C (Fig. 7A), indicating the temperature dependence of the structural changes in the mature domain of the WF146 protease. These temperature-dependent structural changes may account for the slow maturation of the enzyme at low temperatures (Fig. 5C).

We speculated that the reversible conversion between the proform and N*-IE is attributed to the temperature- and/or Ca\(^{2+}\)-induced structural changes in these enzyme forms. To test this possibility, the far-UV CD spectra of pro-WT were monitored in Ca\(^{2+}\)-free buffer at different temperatures. Pro-WT showed a significant change in secondary structure as the temperature increased from 25 °C (remained as the proform, curve 1, Fig. 7C) to 60 °C (converted to N*-IE, curve 2, Fig. 7C). A subsequent temperature shift from 60 to 25 °C (re-synthesis of the proform, curve 3, Fig. 7C) resulted in another
Maturation of Thermostable WF146 Protease

FIGURE 8. Proposed model for multiple maturation pathways of the WF146 protease. The maturation of the WF146 protease can proceed either through a cis-processing reaction-initiated pathway (steps 1 and 2) or a trans-processing reaction-initiated pathway (steps 1' and 2'). Active WF146 protease that matured and heterogeneous proteases are capable of catalyzing (+) the steps 2, 1', and 2' to accelerate the maturation process (see “Discussion” for details). The active site is indicated by a star. 

change in secondary structure. The spectral curve showed a similar profile to that of the initial sample of pro-WT at 25 °C; however, the mean residue ellipticity values of the re-synthesized sample were lower than those of the initial sample (curves I and 3, Fig. 7C), most likely because the N*-I molecule were not fully re-synthesized to the proform molecules (lane S3, Fig. 6B). In addition, N*-I5 was fully degraded by protease K under Ca2+-free conditions (data not shown), indicating that N*-IE contains at least four Ca2+-binding sites, Ca2+-free conditions, and, thus, may accelerate the whole maturation process. Such an autocatalytic pathway, except for the truncation of the linker peptide, is generally employed by subtilases for maturation (6). In the cases of subtilisins E (22) and BPN' (23), after the cis-processing reaction the newly liberated N terminus of the mature domain moves away from the active site and contributes to the formation of a Ca2+-binding site, which in turn traps the mature domain in a stable conformation (24). Unlike subtilisins E and BPN' but similar to the WF146 protease, some subtilases, such as Tk-subtilisin (8) and protease CDF (10), have a linker peptide between the core domain of the N-terminal propeptide and the mature domain, and their maturation processes include the truncation of the linker peptide. A similar maturation process has also been reported for linker peptide-containing kumamolisin-As (25, 26). In Tk-subtilisin, the binding of Ca2+ to a unique Ca-7 site on the mature domain is required to promote the cis-processing reaction, which is followed by truncation of the structurally disordered linker peptide (8). The Ca-7 site in Tk-subtilisin is not found in the WF146 protease, and the maturation process of WF146 protease differs from that of Tk-subtilisin in that its cis-processing reaction can occur under Ca2+-free conditions. The resulting complex N*-I5, however, is unable to proceed to the truncation and degradation step but is capable of re-synthesis of the proform. In contrast, the proform of the WF146 protease can complete the whole maturation process in the presence of Ca2+. Although the WF146 protease lacks a Ca2+-binding site that corresponds to the Ca-7 site of Tk-subtilisin, this protease contains at least four Ca2+-binding sites, as the ligand residues of the Ca-1, Ca-2, Ca-4, and Ca-5 sites in sphericase (27) are absolutely conserved in the WF146 protease (data not shown). The binding of Ca2+ not only allows the proform of WF146 protease to fold into a native-like structure for cis-processing of N* but also leads to the structural disorder of the linker peptide in N*-IWT for truncation. Obviously, the structural disorder and truncation of the linker peptide can prevent the re-synthesis of the proform, allowing the maturation process to proceed completely. For the linker peptide-lacking subtilisins E and BPN’, the re-synthesis reaction is prevented by the Ca2+-induced movement of the newly liberated N terminus of the mature domain from the active site. In this context, despite the differences in locations of the Ca2+-binding sites, Ca2+-induced structural reorganization seems to be a common strategy employed by subtilases for proper maturation.

DISCUSSION

Our results suggest that the autocatalytic maturation of the WF146 protease can proceed via two pathways; that is, one initiated by cis-processing of the core domain (N*) of the N-terminal propeptide and the other initiated by trans-processing of the linker peptide between N* and the mature domain. Moreover, unlike most subtilases, the WF146 protease matures not only autocatalytically but also hetero-catalytically.

Autocatalytic Maturation Pathway Initiated by Cis-processing Reaction—In this pathway N* is cis-processed by the active site of the mature domain to generate the autoprocessed N*-IWT complex (Fig. 8, step 1). Subsequently, the linker peptide is truncated, accompanied by complete degradation of N* (Fig. 8, step 2). Active WF146 protease that matures earlier may trigger an exponential cascade by catalyzing the truncation of the linker peptide and the degradation of N* intermolecularly and, thus, may accelerate the whole maturation process. Such an autocatalytic pathway, except for the truncation of the linker peptide, is generally employed by subtilases for maturation (6). In the cases of subtilisins E (22) and BPN’ (23), after the cis-processing reaction the newly liberated N terminus of the mature domain moves away from the active site and contributes to the formation of a Ca2+-binding site, which in turn traps the mature domain in a stable conformation (24). Unlike subtilisins E and BPN’ but similar to the WF146 protease, some subtilases, such as Tk-subtilisin (8) and protease CDF (10), have a linker peptide between the core domain of the N-terminal propeptide and the mature domain, and their maturation processes include the truncation of the linker peptide. A similar maturation process has also been reported for linker peptide-containing kumamolisin-As (25, 26). In Tk-subtilisin, the binding of Ca2+ to a unique Ca-7 site on the mature domain is required to promote the cis-processing reaction, which is followed by truncation of the structurally disordered linker peptide (8). The Ca-7 site in Tk-subtilisin is not found in the WF146 protease, and the maturation process of WF146 protease differs from that of Tk-subtilisin in that its cis-processing reaction can occur under Ca2+-free conditions. The resulting complex N*-I5, however, is unable to proceed to the truncation and degradation step but is capable of re-synthesis of the proform. In contrast, the proform of the WF146 protease can complete the whole maturation process in the presence of Ca2+. Although the WF146 protease lacks a Ca2+-binding site that corresponds to the Ca-7 site of Tk-subtilisin, this protease contains at least four Ca2+-binding sites, as the ligand residues of the Ca-1, Ca-2, Ca-4, and Ca-5 sites in sphericase (27) are absolutely conserved in the WF146 protease (data not shown). The binding of Ca2+ not only allows the proform of WF146 protease to fold into a native-like structure for cis-processing of N* but also leads to the structural disorder of the linker peptide in N*-IWT for truncation. Obviously, the structural disorder and truncation of the linker peptide can prevent the re-synthesis of the proform, allowing the maturation process to proceed completely. For the linker peptide-lacking subtilisins E and BPN’, the re-synthesis reaction is prevented by the Ca2+-induced movement of the newly liberated N terminus of the mature domain from the active site. In this context, despite the differences in locations of the Ca2+-binding sites, Ca2+-induced structural reorganization seems to be a common strategy employed by subtilases for proper maturation.

Auto- and Hetero-catalytic Maturation Pathway Initiated by the Trans-processing Reaction—The maturation of the WF146 protease can also be initiated via trans-processing of the proform linker peptide (Fig. 8, step 1’). Then the processed N-terminal propeptide will be degraded, releasing the active mature form (Fig. 8, step 2’). In the autocatalytic maturation process, however, the initiation of trans-processing reaction relies on the presence of active WF146 protease coming from the cis-processing reaction-initiated pathway. Most likely, the cis-processing reaction-initiated pathway plays the dominant role at early stages of the autocatalytic maturation process. With the accumulation of active mature enzyme, the trans-processing reaction-initiated pathway will become evident at late stages.
the presence of heterogeneous proteases, both the cis- and trans-processing reactions will occur at early stages. Moreover, heterogeneous proteases can accelerate the conversion of the autoprocessed complex to the mature form by processing the linker peptide and degrading the N-terminal propeptide (Fig. 8, step 2). Nevertheless, the occurrence of the hetero-catalytic maturation in vivo depends on the presence of other proteolysis-producing microorganisms living around Bacillus sp. WF146.

The linker peptide of the WF146 protease comprises a proteolytically sensitive region, enabling the trans-processing reaction to occur. Similarly, the protease CDF proform with a linker peptide was trans-processed to the mature form by mature protease CDF and proteinase K (10). Crystal structure analyses of Tk-subtilisin and kumamolisin revealed that the linker peptides are disordered (8, 28); however, whether these enzymes mature via a trans-processing reaction-initiated pathway remains unknown. Pro-Tk-subtilisin was found to be resistant to chymotryptic digestion, although its linker peptide possesses the potential cleavage sites that are preferred by chymotrypsin (29). In that study, however, the digestion reaction was performed at 30 °C, and thus, it is unclear whether the linker peptide of the thermophilic Tk-subtilisin will become susceptible to proteolysis at high temperatures.

Trans-processing of the N-terminal propeptide has also been reported for subtilases that lack linker peptides. Hu et al. (30) proposed that the propeptide of one pro-subtilisin E molecule is the template for the refolding of the mature sequence of the second, and in turn, the hydrolytic process proceeds intermolecularly as well. Indeed, trans-processing can take place through an intramolecular interaction between two mutant pro-subtilisin E molecules at high protein concentrations (31); however, the addition of active subtilisin to autoprocessed pro-subtilisin E in trans does not facilitate enzyme maturation but rather promotes rapid autodegradation (24). The proforms of subtilisin BPN’ (9) and subtilisin Carlsberg (32) can be processed to their mature forms in trans by active subtilisins. The junction between the N-terminal propeptide and the mature domain of these linker peptide-lacking subtilases directly interacts with the active site cleft in a substrate-like manner to favor the cis-processing reaction (4). Obviously, the occurrence of the trans-processing reaction in these subtilases requires disruption of the interaction between the junction and the active site cleft to render the junction available to another active site for proteolytic attack, but the energetic cost of disrupting the interaction is large (23). Therefore, even if the trans-processing reaction can occur, the cis-processing pathway will usually predominate during the maturation process of these subtilases that lack a linker peptide (33).

**Mechanism of Cis-processing/Re-synthesis of the Proform—** Serine proteases are known to follow a two-step reaction (acylation and hydrolysis) mechanism to cleave peptide bonds (3, 34). Based on this principle and our experimental results, we propose a mechanism for the cis-processing/re-synthesis of the WF146 protease proform (Fig. 9). In the presence of Ca$^{2+}$, the catalytic Ser$^{249}$ residue of the Ca$^{2+}$-bound proform attacks the Ala$^{13}$-Glu$^{12}$ peptide bond to form a covalently linked acyl-enzyme intermediate via an ester bond (acylation), and this is followed by a nucleophilic attack on the ester bond by water (hydrolysis) to form the autoprocessed N*-IWT complex. The hydrolysis reaction requires the newly liberated Glu$^{12}$ amino group to move away from the ester bond, allowing a water molecule to approach the bond for hydrolysis. The cleavage of the Ala$^{13}$-Glu$^{12}$ bond is accompanied by a subtle structural adjustment of N*-IWT, resulting in the release of the linker peptide from the active site. Therefore, the amino group of Glu$^{12}$ is unable to attack the ester bond for re-ligation of the Ala$^{13}$-Glu$^{12}$ peptide bond (aminolysis). Under Ca$^{2+}$-free conditions and at high temperatures (e.g. 60 °C), the proform is converted to a covalently linked acyl-enzyme intermediate (N*-I$^E$) that adopts a non-native conformation. It is unlikely that N*-I$^E$ can be further hydrolyzed to an autoprocessed complex. If the hydrolysis reaction has taken place, the generated autoprocessed complex will adopt a native-like conformation upon binding of Ca$^{2+}$ and will then follow the maturation pathway of N*-IWT to yield mature enzyme. Actually, N*-I$^E$ has converted to the proform rather than the mature form in the presence of Ca$^{2+}$ or at low temperatures. In contrast, N*-IWT is unable to convert to the proform under the same conditions. In addition, the structure of N*-I$^E$ is not identical to that of the autoprocessed complex N*-I$^{249C}$ under Ca$^{2+}$-free conditions. It is, therefore, most likely that N*-I$^E$ exists as a covalently linked acyl-enzyme intermediate with non-native conformation, and the linker peptide still shields the active site to prevent a water molecule from approaching the ester bond for hydrolysis.
binding of Ca\(^{2+}\) or being shifted to low temperatures, N*\(^{-}\)I\(^{E}\) undergoes a structural adjustment, which favors the amino group of Glu\(^{12}\) to attack the ester bond for re-ligation of the peptide bond Ala\(^{-13}\)-Glu\(^{-12}\) (aminolysis). Although the molecular details of this re-ligation process remains to be elucidated, a plausible mechanism is that the reversible conversion between the proform and N*\(^{-}\)I\(^{E}\) is controlled by the accessibility of the newly liberated amino group of Glu\(^{12}\) to the ester bond, which is attributed to the temperature- or Ca\(^{2+}\)-induced structural changes in the proform and N*\(^{-}\)I\(^{E}\).

Re-synthesis of the proform has been reported for mutant kumamolisin-As that contains an engineered active site (E78H/D164N) (26). The major difference between the mechanism proposed for E78H/D164N and that for the WF146 pro tease is that the ligation reaction starts with a noncovalently associated autoprocessed complex for E78H/D164N but begins with a covalently linked acyl-enzyme intermediate for the WF146 pro tease. In addition, the ligation reaction of E78H/D164N is triggered by pH-induced deprotonation of the His\(^{78}\) residue, whereas the ligation reaction of the WF146 pro tease is caused by temperature- or Ca\(^{2+}\)-induced structural changes. In both cases, however, the newly liberated N-terminal amino group of the linker peptide resides in the vicinity of the active site to facilitate the ligation reaction. In this context the linker peptides of E78H/D164N and the WF146 pro tease play similar roles in regulation of the cis-processing/re-synthesis equilibrium.

In summary, this study reveals that the proform of the thermophilic WF146 pro tease is intrinsically able to mature both auto- and hetero-catalytically. In addition, we found that under Ca\(^{2+}\)-free conditions, the proform could be cis-processed to a unique intermediate capable of re-synthesis of the proform. The fact that high temperature and Ca\(^{2+}\) binding induce a shift of the cis-processing/re-synthesis equilibrium to favor the maturation process reflects an adaptation strategy of this thermophilic pro tease.

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Maturation of Thermostable WF146 Protease

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