Myroilysin Is a New Bacterial Member of the M12A Family of Metzincin Metallopeptidases and Is Activated by a Cysteine Switch Mechanism*

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Proteases play important roles in all living organisms and also have important industrial applications. Family M12A metalloproteases, mainly found throughout the animal kingdom, belong to the metzincin protease family and are synthesized as inactive precursors. So far, only flavastacin and myroilysin, isolated from bacteria, were reported to be M12A proteases, whereas the classification of myroilysin is still unclear due to the lack of structural information. Here, we report the crystal structures of pro-myroilysin from bacterium Myroides sp. cslb8. The catalytic zinc ion of pro-myroilysin, at the bottom of a deep active site, is coordinated by three histidine residues in the conserved motif HExxHxxGxxHH; the cysteine residue in the pro-peptide coordinates the catalytic zinc ion and inhibits myroilysin activity. Structure comparisons revealed that myroilysin shares high similarity with the members of the M12A, M10A, and M10B families of metalloproteases. However, a unique “cap” structure tops the active site cleft in the structure of pro-myroilysin, and this “cap” structure does not exist in the above structure-reported subfamilies. Further structure-based sequence analysis revealed that myroilysin appears to belong to the M12A family, but pro-myroilysin uses a “cysteine switch” activation mechanism with a unique segment, including the conserved cysteine residue, whereas other reported M12A family proteases use an “aspartate switch” activation mechanism. Thus, our results suggest that myroilysin is a new bacterial member of the M12A family with an exceptional cysteine switch activation mechanism. Our results shed new light on the classification of the M12A family and may suggest a divergent evolution of the M12 family.

Proteases play important roles during the life of all organisms, including microbes (1). Intracellular proteases are of great importance for various metabolic processes, such as protein turnover, sporulation, and differentiation (2,3). Extracellular proteases usually hydrolyze proteins in the environment to provide nutrients to the bacteria (4–7), although some are thought to be involved in pathogenesis (8). Because extracellular proteases are easily obtained, they are also widely applied in various industries and medical fields, including detergent, leather, food, waste treatment, diagnosis of illness, and pharmaceuticals (9–13).

Metalloproteases, a group of important proteases, require a divalent metal cation (usually zinc ion) for activity. The metal ion in metalloprotease is coordinated by several amino acid residues, commonly His, Glu, Asp, or Lys (14). Zinc-dependent metalloproteases contain a signature motif HExxH, in which the two histidine residues function as the first and second zinc ligands and the glutamate residue plays an outstanding role in catalysis (15). When the third zinc ligand is a glutamate residue, zinc-dependent metalloproteases are called gluzincins, a group that consists of exopeptidases and endopeptidases (14). If the third zinc ligand is a histidine or asparagine residue in the elongated motif HExxHx(G)xx(H/D), the conserved glycine residue is indispensable for forming a β-turn to bring the three zinc ligands together (16). These types of zinc-dependent metalloproteases are known as metzincins because of a conserved methionine residue in a Met-turn (such as SIMHY) that underlies the active site (17) to form a structure that acts as a “hydrophobic pillow.” All metzincins are endopeptidases and are generally synthesized as inactive precursors (14). In some members of the metzincins, such as the M8, M10, M11, and M12 families, a conserved cysteine residue in the pro-peptide can interact with the catalytic zinc ion to prevent the binding of a water molecule and inactivate the protease. This inhibition mechanism is known as a “cysteine switch” (14).

The M12 family is the second largest family of metzincins and can be further divided into two subfamilies: subfamilies M12A and M12B (14). Subfamily M12A is known as the astacin subfamily (18). These metalloproteases from the M12A subfamily contain a conserved aspartate residue (Asp-21) in the pro-peptide, and their activation mechanism is therefore called “aspartate switch” (19). Proteases in the M12A family are mainly found throughout the animal kingdom and only rarely found in bacteria. Flavastacin (20–22) is the only confirmed bacterial protease in this family. Members in subfamily M12B

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The atomic coordinates and structure factors (codes 5CZW and SGWD) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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(also called the repolyisin family) employ a cysteine switch mechanism (14). Repolyisin, found in snake venom, is the representative member. A cysteine switch mechanism for the pro-peptide is thought to operate in the inhibition of repolyisin. Proteases in the M12B subfamily are also widely distributed throughout the animal kingdom.

The Myroides sp. cslb8, isolated from silkworm excrement in our laboratory, secretes a 25-kDa protease. Mass spectrometry analysis and BLAST analysis of the amino acid sequence indicated that this protease shares high sequence identity with the reported myroilysin from Methanolobus profundi D25, which was classified as an M12A subfamily protease based on a sequence alignment (23). However, myroilysin is classified into the M10B family in the MEROPS (24) peptidase database without structural information.

The M10 family, like the M12 family, is synthesized as inactive precursor and can be divided into three subfamilies: M10A, M10B, and M10C. The M10A subfamily proteases are mainly found throughout the animal kingdom and activated through a cysteine switch mechanism. The best known examples are the eukaryotic matrix metallopeptidases (MMPs) (25). The M10A proteases are also present in plants and bacteria, but the non-animal M10A proteases do not usually employ a cysteine switch activation mechanism (14). Karilysin is a typical bacterial M10A member; it is expressed as a proenzyme with an asparagine residue in the pro-peptide that may act in a similar manner to proastacin (25, 26). Proteases in the M10B subfamily are mainly from Proteobacteria, including the well studied serralysin (27).

Sequence analysis revealed a cysteine (Cys-26) in the predicted pro-peptide and a typical M-turn (SIMHY) in the amino acid sequences of the myroilysin from Myroides sp. cslb8. We therefore investigated the question: Should myroilysin belong to the M12 or the M10 family?

To elucidate the catalytic mechanism of myroilysin and clarify the classification of myroilysin, genes encoding myroilysin and pro-myroilysin from Myroides sp. cslb8 were cloned and expressed. The 1.89 and 1.6 Å crystal structures of pro-myroilysin were resolved. The structure comparison revealed that this protease shares high sequence identity with the peptidase from Myroides sp. cslb8. We therefore investigated the question: Should myroilysin belong to the M12 or the M10 family?

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**Results and Discussion**

**Isolation and Characterization of Strain cslb8**—Strain cslb8, an orange colony with the largest clear zone on the skim milk plate, was selected for further study. The cells of cslb8 were Gram-negative and rod-shaped. These cells grow well at pH 7–10, with an optimum pH for growth at 7. No acid was produced from glucose, lactose, sucrose, and fructose. To deter-

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3 The abbreviations used are: MMP, matrix metallopeptidase; BisTris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol; r.m.s.d., root mean square deviation(s); PDB, Protein Data Bank; M-turn, Met-turn.
Crystal Structure of Pro-myroilysin

The pro-myroilysin monomer comprises 242 residues and is composed of a pro-peptide consisting of residues 1–37, an N-terminal domain consisting of residues 38–151 and residues 229–242, and a C-terminal domain consisting of residues 152–228. The overall structure indicates that pro-myroilysin is a spherical molecule (Fig. 2, A and C). The crystal structure of pro-myroilysin from the 0.1 M HEPES-NaOH, pH 7.5, 1.4 M sodium citrate condition was resolved to 1.89 Å. The topology of pro-myroilysin is shown in Fig. 2B. The crystal belongs to the $P_2_1$ space group with cell dimensions $a = 72.2$, $b = 35.5$, $c = 93.8$ Å. There are two almost identical molecules of pro-myroilysin with root mean square deviations (r.m.s.d.) of 0.35 Å in the asymmetric unit. In the pro-myroilysin molecule, 20% of residues form five $\beta$-strands. The pro-peptide in this model is almost perpendicularly against each other and cover the active site.

The three-dimensional structure of pro-myroilysin from the 0.1 M BisTris, pH 6.5, 40% PEG 4000 condition was resolved at 1.6 Å with $R_{work}$ of 19.1% and $R_{free}$ of 23.0%. The crystal belongs to the $P_2_1$ space group with cell dimensions $a = 51.2$, $b = 35.1$, $c = 64.3$ Å. There is only one molecule of pro-myroilysin in the asymmetric unit. In the pro-myroilysin molecule, 20% of residues form seven $\alpha$-helices, and 7% of residues form four $\beta$-strands (Fig. 2C). Notably, the electron density is invisible for the fragment from residue Thr-36 to Arg-44. The topology of pro-myroilysin is shown in Fig. 2D.

A comparison of the structures obtained from these two conditions revealed that they are almost identical, with an r.m.s.d. of 0.35 Å, but some differences between these two structures were still observed, including the regions from 19 to 23, from 153 to 160, and from 214 to 224 and missing residues 36–44. These differences may stem from the crystal packing caused by intramolecular and intermolecular interactions.

Active Site—The N-terminal domain and the C-terminal domain form a deep V-shaped cleft, and the active site is located at the bottom of the cleft (Figs. 2 and 3). In the long and deep active site cleft, the catalytic zinc ion at the bottom is coordinated in a triangular pyramidal geometry by three NE2 atoms of His-140 (2.3 Å), His-144 (2.1 Å), and His-150 (2.1 Å), belonging to the signature motif HEXHHXXGXXH. The zinc cation is also coordinated by the SG atom of the side chain of the cysteine residue Cys-26 in the pro-peptide, with a distance of 2.3 Å. The catalytic residue Glu-141, following the first histidine zinc ligand (His-140), is not involved in coordinating the zinc ion, but the OE2 atom of Glu-141 forms a hydrogen bond (3.0 Å) with the SG atom of Cys-26. The side chains of His-140, Glu-141, and His-144 from the central helix $\alpha_6$ project into the deep active site cleft, and the $\alpha_6$ helix extends to Gly-147, where it turns sharply to bring the third coordinator (His-150) of zinc ion to the active site (Fig. 3A). The following Glu-151, the direct neighbor of the third zinc ligand His-150, is thought to be strictly conserved in the members of astacins (32).

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**Table 1**

| Data collection and refinement statistics | Peak | Native |
|---|---|---|
| **Crystallization conditions** | 0.1 M HEPES-NaOH, pH 7.5, 1.4 M sodium citrate | 0.1 M BisTris, pH 6.5, 40% PEG 4000 |
| **Data collection** | | |
| Wavelength (Å) | 1.2816 | 0.9791 |
| Temperature (K) | 100 | 100 |
| Crystal-to-detector distance (mm) | 200 | 200 |
| Rotation range per image (degrees) | 1 | 1 |
| Total rotation range (degrees) | 540 | 360 |
| Space group | $P_2_1$, $P_2_1$ |
| Unit-cell parameters | $a, b, c$ (Å) | 72.2, 35.5, 93.8 |
| No. of reflections used | 35,832 | 27,216 |
| Multiplicity | 10.6 | 7.4 |
| Completeness (%) | 98.6 (91.0) | 99.9 (100) |
| Free (%) | 22.38 | 23.00 |
| **Structure refinement** | | |
| Total no. of atoms | 4,000 | 2,056 |
| No. of reflections used | 35,832 | 27,216 |
| $R_{work}$ (%) | 18.15 | 19.09 |
| $R_{free}$ (%) | 22.38 | 23.00 |
| r.m.s.d. bonds (Å) | 0.0067 | 0.0062 |
| r.m.s.d. angles (degrees) | 0.855 | 0.857 |
| Ramachandran plot (%) | | |
| Favored | 98.5 | 96.44 |
| Allowed | 1.5 | 3.56 |
| Outlier | 0 | 0 |

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*a* The highest resolution shell is shown in parenthesis.
In the other signature motif, SIMHY (residues 204–208), the oxygen atom of the Ile-205 main chain carbonyl group forms a specific interaction with the ND1 atom of the first zinc ligand His-140 through a hydrogen bond (2.7 Å) (Fig. 3B) and is involved in structure stability. However, the side chain of Tyr-208 faces away from the catalytic zinc (Fig. 3), whereas the corresponding tyrosine is engaged in zinc and substrate binding and stabilization in mature astacin and serralyisin (27, 33, 34).

Structure Comparison of Pro-myroilysin with Homologues from M12A, M10A, and M10B Subfamilies—A structure similarity search performed with the atomic coordinates of pro-myroilysin using the DALI server yielded the M12 family pro-astacin (PDB code 3LQ0) (19), pro-meprin (PDB code 4GWM) (35), choriolysin (PDB codes 3VTG and 3LQB) (36), M10 family prokarilysin (PDB code 4R3V) (37), macrophage metalloelastase (PDB code 1UTT) (38), and M12 family astacin (PDB code 1AST) (16), among others.

A structural comparison of pro-myroilysin with homologues from M12A, M10A, and M10B subfamilies was also performed. Pro-myroilysin superimposes with the M12 family proteases pro-astacin (PDB code 3LQ0) and pro-meprin (PDB code 4GWM) with an r.m.s.d. of 3.2 Å (for 178 target pairs) and 2.9 Å (for 172 target pairs) and shares amino acid sequence identities of 20 and 21%, respectively. Fig. 4A shows that the N-terminal β1−5 strands of pro-myroilysin partially superimpose onto the corresponding β strands of pro-astacin and pro-meprin homodromously; helices α4, α6, and α9 also partially superimpose onto the corresponding helices of pro-astacin and pro-meprin homodromously. However, the β5 strand of pro-myroilysin is much shorter than the corresponding β-strand of pro-astacin and pro-meprin and instead forms a loop structure protruding outside of the protein. This may be caused by the helix α5 located between β4 and β5. There are also many structural differences between the structures; for example, the α3 helix
could not superimpose onto the corresponding helix. The most remarkable structural differences are present at the C terminus. The C-terminal domain of pro-myroilysin consists of coils and two helices (α7 and α8; purple in Fig. 2, A and C); the two helices form the unique cap structure situated above the active site. The C-terminal domains of pro-astacin and pro-meprin have four or five helices and three β strands, but there is no obvious cap structure above the active sites.

Moreover, pro-myroilysin superimposes onto the M10A family protease pro-karilysin (PDB code 4R3V) with an r.m.s.d. of 2.7 Å (for 154 target pairs); the amino acid sequence identities were 15 and 21%, respectively. As shown in Fig. 4B, the structure of pro-myroilysin also shows similar structural similarity to pro-karilysin and the catalytic domain of serralysin. The helices α4, α6, and α9 of pro-myroilysin partially superimpose onto the corresponding helices of pro-karilysin and the catalytic domain of serralysin homodromously, and β1–5 strands also superimpose well and are located on the surface of both molecules. The C-terminal domain of pro-karilysin and the catalytic domain of serralysin also do not contain a cap structure. Overall, the superimposition of pro-myroilysin with M12A, M10A, and M10B family proteases showed that the N-terminal domain of pro-myroilysin superimposes well with them, whereas the C terminus is evidently structurally different, particularly the cap structure.

Detailed structural and sequence analyses were also performed. As mentioned above, the conserved Gly-147 (equivalent to Gly-99 of astacin and Gly-181 of serralysin) and the M-turn SIMHY are also present, whereas the Tyr-208 (equivalent to Tyr-149 in astacin and Tyr-216 in the serralysin, involved in zinc binding) points away from the zinc in pro-myroilysin (Fig. 3). In addition, the Tyr-239 of pro-myroilysin also perfectly matches the Tyr-194 of astacin and Tyr-246 of serralysin, which are involved in locking the C-terminal helix to the molecular moiety. The 90-loop of pro-myroilysin is exactly equivalent to the loop connecting strands β2 and β3 in astacin and serralysin.

It has been reported that there are two disulfide bonds in astacin and that these two bonds are likely to be conserved among all astacins and contribute to shaping the active site cleft (39). However, no disulfide bond is present in pro-myroilysin, as in serralysin and pro-karilysin.

Inhibition Mechanism—For pro-astacin, pro-meprin, and pro-karilysin, one striking difference could also be observed from pro-myroilysin. The pro-peptides of pro-astacin, pro-meprin, or pro-karilysin, containing 34, 37, or 14 residues, run through the cleft, and a side chain atom of a conserved aspartate residue (Asp-21, Asp-52, or Asp-25) of the pro-peptide is anchored to the catalytic zinc ion to replace the zinc-binding solvent molecule (Fig. 4, C–E). This is the “aspartate switch” activation mechanism (19). For pro-myroilysin, the pro-peptide consisting of 37 residues runs through and occupies the active site cleft to prevent access to peptide substrates. Instead of an aspartate residue, the side chain sulfur atom of Cys-26 of the pro-peptide coordinates the catalytic zinc ion to expel the catalytic water molecule from the activity site, allowing the inhibition of pro-myroilysin catalysis and its so-called “cysteine switch” mechanism (Figs. 3 and 4 (C–E)). Other than this difference, the direction of binding in the pro-peptide of the active site cleft of pro-myroilysin is the same as that seen in the pro-astacin structure (Fig. 4A).

Interestingly, the segment including the conserved “cysteine switch” is unique in pro-myroilysin; the AKVCKDV motif has never been reported, whereas PRCGPD is conserved in MMPs, PKMCGV in ADAMs (a disintegrin and metalloproteinase), HRCIHD in leishmanolysins, and CG in pappalysins (17).
PISA (40) was used to analyze the interactions between the pro-peptide and the mature peptide of pro-myroilysin. The pro-peptide main chain, consisting of 37 residues, extends along the spherical molecule surface of the mature peptide to the active site pocket. Starting at Arg-9, the residues bind the surface of the mature enzyme; from Ala-23, the main chain runs through the active site pocket. The pro-peptide of pro-myroilysin is firmly bound to the mature peptide through several intramolecular interactions, such as hydrogen bonds and salt bridges. Eleven hydrogen bonds are formed between the eight residues (Arg-9, Asp-15, Val-25, Cys-26, Lys-27, Asp-28, Asp-35, and Pro-36) of the pro-peptide and the 11 residues (Gly-38, Ala-39, Ala-100, Ser-102, Asn-116, Glu-117, His-150, Tyr-173, Asn-175, Tyr-176, and Tyr-190) of the mature peptide (Fig. 5A); two salt bridges form between the Cys-26 of the pro-peptide and Tyr-170 and Tyr-208 of the mature peptide (Fig. 5B). In addition, the cysteine residue of the pro-peptide itself (Cys-26) is tightly bound to the catalytic zinc ion (Fig. 3). Therefore, the pro-peptide is firmly fixed in the active site pocket.

In the reported mature enzymes of the M10 and M12 family of proteases, the catalytic water molecule is anchored by the glutamate residue following the first histidine zinc ligand (41) and is thought to be responsible for the nucleophilic attack to the carbon atom of the carbonyl group of the substrate peptide bond (14). However, in pro-myroilysin the catalytic water molecule is absent, and instead a cysteine residue (Cys-26) in the pro-peptide coordinates the zinc ion. Cys-26 also forms a hydrogen bond with the catalytic Glu-141 residue (Fig. 3). Thus, the presence of Cys-26 expels the catalytic water molecule away from the active site and results in inhibition of protease activity.

A Unique Cap Structure in Pro-myroilysin May Be Involved in Substrate Binding—Structure-based sequence alignment of pro-myroilysin with other proteases with Z scores > 10 indicate that these proteases belong to the M10 or M12 subfamily, especially the M12A, M10A, and M10B subfamilies. However, the insert between the two conserved motifs of pro-myroilysin (HEXHXGXH and M-turn SIMHY) are much longer than in other homologs (Fig. 6). This fragment (residues 160–193) includes the helices α7 and α8, forming the cap in the pro-myroilysin structure (Fig. 2, A and C).

Work by Chen showed that myroilysin has broad specificity and high elastinolytic activity, indicating that the active cleft of myroilysin is opened before the substrate accesses it (23).
unique cap structure in pro-myroilysin, which does not exist in the other structure-reported members of the M12 or M10 family, may help to stabilize the pro-peptide through hydrogen bonds (Fig. 5A). Tyr-170 and Tyr-190 are two unique amino acid residues in the cap structure. The oxygen atom of Tyr-190 forms a hydrogen bond (2.9 Å) with the third histidine zinc ligand (His-150), and therefore the Tyr-190 residue seems to stabilize the conformation of the third histidine and the pro-peptide of pro-myroilysin (Fig. 5A). Furthermore, the Tyr-170 residue in the cap could also stabilize the pro-peptide through water-mediated hydrogen bonds (Fig. 5B).

In our pro-myroilysin structure, the cap domain covers the pro-peptide and forms a tunnel that holds the pro-peptide inside (Fig. 5C). If the tunnel exists throughout the entire catalytic cycle, the substrate must be inserted into the tunnel for cleavage. However, because the tunnel is not wide enough to directly let the substrate get into the tunnel, we hypothesized that there is a conformational change to expose the active site, with the cap moving away from the active site after the N-terminal pro-peptide of pro-myroilysin is proteolytically removed.

In addition, it has been reported that the Glu-103 of astacin, the amino acid residue just after the third zinc-binding His in the HEXXHXXGXXH motif, is important for structural stability due to its water-mediated salt bridge to the N-terminal Ala-1 after the pro-peptide is cleaved (42). The main chain of astacin rotates 180° around the Ψ main chain angle of the new N-terminal residue to allow the N terminus to bury into the mature enzyme body to maintain the structural features. The removal of the pro-segment would offer sufficient space for the activation domain to enclose its substrate (39). This Glu is thought to be the family-specific residue of astacin family (M12A) proteases, whereas the corresponding residue is a proline or serine in the serralysin family of protease (M10B) or MMPs (M10A) (17, 32). Inspection of the mature N termini of representative astacin family members also showed that N-terminal residues are almost exclusively alanine or asparagine (39). Glu-151 is the corresponding amino acid residue in the HEXXHXXGXXH motif of pro-myroilysin. However, the Glu-151 is far from the Gly-38, the first amino acid residue of the mature myroilysin (23) in the structure of pro-myroilysin. Instead, Glu-151 can form hydrogen bonds with Thr-195 and Gln-196 just after the α7 and α8 helices (cap structure), which do not exist in astacin, and keeps the Glu-151 from the Gly-38 in the pro-myroilysin (Fig. 7). This may imply that the unique cap structure in pro-myroilysin might change the structure arrangement. However, the real role of the cap structure in the mature form of myroilysin is still unknown.

The structure of mature myroilysin would answer the question, but unfortunately, we failed to obtain diffractable myroilysin crystals of native mature myroilysin from the culture.
supernatant of csbl8 and failed to obtain myroilysin expressed in *E. coli*. This may imply that myroilysin with proteolytic activity is toxic to *E. coli* cells.

Structure and Sequence Analysis Suggest That Myroilysin Is a Bacterial M12 Family Protease—The M10 and M12 families of proteases are two major families of metzincins. They share quite high sequence similarity, zymogen activation, and catalytic mechanisms, including the signature conserved motifs HEXXHXXGG (H/D) and Met-turn (14). The M12 family is the second largest family in the metzincins, and proteases in this family are mainly found throughout the animal kingdom and rarely in bacteria. Although quite a few structures from the M12 family have been solved, no structural information is available for M12 family proteases from bacteria. The flavastacin from *Flavobacterium meningosepticum* (20–22) is the only reported bacterial protease in this family. Myroilysin is a newly identified metazincin from *M. profundi* D25, and amino acid sequence analysis showed that it belongs to the M12A family of proteases (23). However, myroilysin is classified in the M10B family in the peptidase database MEROPS.

Here, we report the crystal structures of pro-myroilysin in two different crystal forms from *Myroides* sp. csbl8. A structural comparison of pro-myroilysin with some M12 and M10 family proteases, including astacin, meperin, karilysin, and serralyasin, is shown in Fig. 4. The overall structural comparison with these M12 and M10 family proteases showed that the N-terminal domain of pro-myroilysin superimposes well, whereas the C-terminal domain is structurally different. The signature motif HEXXHXXGG and the M-turn (SIMHY) of pro-myroilysin are strictly conserved (Fig. 4). The Dali server results showed that the structure of pro-myroilysin shares some similarity with M12A, M10A, and M10B family proteases, such as pro-astacin, pro-meperin and serralyasin. The phylogenetic tree of the myroilysin structure-based homologs based on the Dali server (Z > 10) was then constructed. Fig. 8 shows that distinct clusters form for M10 and M12 family proteases; pro-myroilysin is clearly more closely related to M12A family proteases than M10B family proteases or M10A family proteases. The above results have showed a conserved Glu residue just after the third zinc-binding His in the HEXXHXXGXXH motif, which is thought to be a family-specific residue of the astacin family; non-animal M10A proteases do not usually employ a cysteine switch activation mechanism (as in pro-karilysin). We conclude that pro-myroilysin is a bacterial M12 family protease that is toxic to *E. coli* cells.
lysin should belong to the M12A family rather than the M10 family. However, pro-myroilysin also forms a distinct branch with structurally determined M12A family proteases in the phylogenetic tree and has a different activation mechanism (cysteine switch mechanism) from the M12A family. In addition, due to a special cap structure, different N terminus amino acid residues in the mature enzyme, a different binding character of Glu151, and its function in pro-myroilysin, we also conclude that myroilysin should be a new member of the M12A family of proteases or may even form a new M12 subfamily. In summary, our crystal structures and structural comparison with M12 and M10 family proteases contribute new insights into the classification of the M12 metalloprotease family and may imply a divergent evolution of this family.

Materials and Methods

Biological Material and Culture Conditions—Bacterial strain cslb8 was isolated from silkworm feces on the LB medium (1% NaCl, 1% tryptone, and 0.5% yeast extract) with a 2% skim milk plate. Strain cslb8 was inoculated in LB medium on a rotary shaker at 180 rpm at 28 °C for 12 h and then transferred into 1 liter of LB medium at 28 °C for another 12 h. The culture was centrifuged at 8,000 rpm, and the supernatant was then used for protein isolation and purification.

16S rDNA Sequencing—The 16S rDNA gene of CSLB8 was amplified by PCR using the genomic DNA as template with the

FIGURE 7. The conserved Glu-151 just after the third zinc-binding His in the HEXXHXXXGXXH motif of pro-myroilysin interacts with Thr-195 and Gln-196 just after the cap structure. The conserved Glu-151 forms hydrogen bonds with Thr-195 and Gln-196 and distances itself far from Gly-38 (the first amino acid residue in myroilysin) in pro-myroilysin. In contrast, the Ala-1 of proastacin is much closer to the corresponding residue Glu-101 and forms water-mediated salt bridges to the N-terminal Ala-1 after the pro-peptide is cleaved. The pro-peptide, the cap structure, and the rest of pro-myroilysin (including the zinc ion) are in orange, purple, and cyan, respectively. Gly-38, Glu-151, Thr-195, and Gln-196 of pro-myroilysin are rendered in green sticks; the pro-peptide and the rest of pro-astacin are light yellow and gray, respectively. Glu-101 (light gray) and Ala-1 of astacin (light gray) and Ala-1 in pro-astacin (dark gray) are shown as sticks; the zinc ions in pro-astacin and astacin are shown in dark gray and light gray, respectively; water molecules are shown in red.

FIGURE 8. Structure-based phylogenetic tree illustrating the relationship between the pro-myroilysin and its homologs. The data set of these homologs was obtained from DALI by selecting proteins with Z score > 10 when compared with pro-myroilysin. For the sake of clarity, only 46 proteins are shown in the phylogenetic tree. Scale bar, phylogenetic distance. PDB codes and protein names are listed. ZHE1, zebrafish hatching enzyme 1; HCE1, high choriolytic enzyme 1; BMP1, bone morphogenetic enzyme 1; HCE1, high choriolytic enzyme 1; BMP1, bone morphogenetic protein 1; ProMMP-2, pro-matrix metalloproteinase-2; HFC, human fibroblast collagenase; FC-1, fibroblast collagenase 1; MT1-MMP, membrane type 1 matrix metalloproteinase; MMP20, enamelysin; MMP-11, stromelysin-3.
Crystal Structure of Pro-myroilysin

ture supernatant to reach 80% saturation. The precipitate was collected after centrifugation at 15,000 rpm for 30 min. The precipitate was dissolved in 40 mM KH₂PO₄/K₂HPO₄ (pH 7.6) and diluted with an equal volume of 2 M ammonium sulfate solution. After centrifugation, the sample was then loaded onto an octyl-Sepharose 4 fast flow chromatography column (2-mL bed volume) pre-equilibrated with binding buffer (20 mM KH₂PO₄/K₂HPO₄, pH 7.6, and 1 M (NH₄)₂SO₄). After washing with 5 bed volumes of binding buffer, the protein was eluted with a linear gradient of 1 to 0 M ammonium sulfate. The eluted protease was then concentrated by the Amicon Ultra15 centrifugal filter unit with a 10 kDa molecular mass cut-off (Merck Millipore) and further purified by the Sephadex G200 gel filtration column pre-equilibrated with 20 mM Tris-Cl, pH 8.0, 300 mM NaCl. The purified protein was then eluted with the same buffer. The purified protein was then inspected on 12% SDS-PAGE. The protein concentration was measured using the PerkinElmer Life Sciences Lambda 25 UV-visible spectrometer at 280 nm.

After SDS-PAGE separation and gel staining, the single protein band was cut off from the gel; the protein was then sequenced by the Beijing Luhe Technology Co. Ltd. and DNA sequencing, and the correct recombinant plasmids (pET24b_pro-myroilysin) were verified by restriction reaction with a methylation protocol (Hampton Research). Methylacylation was carefully performed by dimethylamine borane complex and formaldehyde, as reported previously (46). The methylated pro-myroilysin was concentrated to ~1 mL using an Amicon Ultra-10 filter (Millipore) and then loaded onto a Superdex 200 column pre-equilibrated with 20 mM Tris-Cl, pH 8.5, 300 mM NaCl, and 10% (v/v) glycerol. The column was then carefully eluted with 1.2 bed volumes of the pre-equilibrated buffer. The elution pattern showed that there was only a single peak, which was further analyzed by SDS-PAGE to check the purity of pro-myroilysin. Pro-myroilysin was pooled together and concentrated to 20 mg/mL for crystallization.

Protein Crystallization—Crystallization was performed with the commercial kits from Hampton Research and Microlytic using the sitting drop vapor diffusion method at 4 and 22 °C. The initial screen yielded flake-like crystals at 22 °C from the 0.1 M BisTris, pH 6.5, 40% PEG 4000 condition and flaky crystals at 4 °C from the following three conditions: 0.1 M HEPES-NaOH, pH 7.5, 1.4 M sodium citrate; 1.6 M sodium citrate; and 0.1 M Tris-Cl, pH 8.5, 1.25 M sodium citrate. Because flake-like crystals at the 0.1 M BisTris, pH 6.5, 40% PEG 4000 condition grow better and need a short time to grow to full size (about 7 days), this condition was chosen for further optimization. A systematic grid screening of precipitant concentrations, pH, and protein concentrations was set up to optimize the crystal. The best condition was then optimized with the commercial additive screening kit (Hampton Research).

Data Collection, Structure Determination, and Refinement—The crystal was carefully loooped out from the crystallization drop and quickly cooled in liquid nitrogen. Data collection was performed at 100 K. X-ray diffraction data sets were collected at beamline BL17U1 of the Shanghai Synchrotron Radiation Facility (47). The crystal from the 0.1 M HEPES-NaOH, pH 7.5, 1.4 M sodium citrate condition was diffacted to 1.89 Å. A full data set of 540 frames was collected at the peak wavelength (1.2816 Å) of zinc ion. Each frame was exposed with a rotation range of 1.0 for 1.2 s. Diffraction data were processed with XDS (48). The structure of pro-myroilysin was solved using the SAS protocol of Auto-Rickshaw: the EMBL-Hamburg automated crystal structure determination platform (49). The input diffraction data were prepared and converted for use in Auto-Rickshaw using programs of the CCP4 suite (50).
mated substructure structure factors) values were calculated using the program SHELEXC (51). Based on an initial analysis of the data, the maximum resolution for substructure determination and initial phase calculation was set to 2.4 Å. Both of the two heavy atoms requested were found using the program SHELXD (52). 84.30% of the model was built using the program ARP/wARP (53, 54). The crystal from the 0.1M BisTris, pH 6.5, 40% PEG 4000 condition was diffracted to 1.6 Å. A data set of 360 frames was collected at wavelength 0.9791 Å. The crystal structure of pro-myroilysin from this condition was solved with the molecular replacement method with Phaser using the 1.89 Å crystal structure of pro-myroilysin (55). The structure model was manually adjusted with Coot (56), and structure refinement was performed with REFMAC and Phenix (57, 58). The models were validated with MolProbity (59). All figures were drawn with PyMOL (60) and TopDraw (61). Data processing and refinement statistics are summarized in Table 1.

Sequence Alignment—Structure-based amino acid sequence alignment of members of the M12 family was performed with the Dali server and then redrawn with ClustalX version 1.81 (62) and GENEDOC (43).

**Author Contributions**—W. W., D. X., and T. R. conceived the study. D. X., J. Z., X. L., and T. R. conducted the experiments. J. H. and all other authors participated in data analysis and wrote the manuscript.

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