Hydrolysis of Insoluble Collagen by Deseasin MCP-01 from Deep-sea Pseudoalteromonas sp. SM9913

COLLAGENOLYTIC CHARACTERS, COLLAGEN-BINDING ABILITY OF C-TERMINAL POLYCYSTIC KIDNEY DISEASE DOMAIN, AND IMPLICATION FOR ITS NOVEL ROLE IN DEEP-SEA SEDIMENTARY PARTICULATE ORGANIC NITROGEN DEGRADATION

Guo-Yan Zhao, Xiu-Lan Chen, Hui-Lin Zhao, Bin-Bin Xie, Bai-Cheng Zhou, and Yu-Zhong Zhang

From the State Key Lab of Microbial Technology, Marine Biotechnology Research Center, Shandong University, Jinan 250100, China

Collagens are the most abundant proteins in marine animals and their degradation is important for the recycling of marine nitrogen. However, it is rather unclear how marine collagens are degraded because few marine collagenolytic proteases are studied in detail. Deseasins are a new type of multidomain subtilases. Here, the collagenolytic activity of deseasin MCP-01, the type example of deseasins, was studied. MCP-01 had broad substrate specificity to various type collagens from terrestrial and marine animals. It completely decomposed insoluble collagen into soluble peptides and amino acids, and was more prone to degrade marine collagen than terrestrial collagen. Thirty-seven cleavage sites of MCP-01 on bovine collagen chains were elucidated, showing the cleavage is various but specific. As the main extracellular cold-adapted protease from deep-sea bacterium Pseudoalteromonas sp. SM9913, MCP-01 displayed high activity at low temperature and alkaline range. Our data also showed that the C-terminal polycystic kidney disease (PKD) domain of MCP-01 was able to bind insoluble collagen and facilitate the insoluble collagen digestion by MCP-01. Site-directed mutagenesis demonstrated that Trp-36 of the PKD domain played a key role in its binding to insoluble collagen. It is the first time that the structure and function of a marine collagenolytic protease, deseasin MCP-01, has been studied in detail. Moreover, the PKD domain was experimentally proven to bind to insoluble protein for the first time. These results imply that MCP-01 would play an important role in the degradation of deep-sea sedimentary particulate organic nitrogen.

Collagens, which distribute in skin, scale, bone, tendon, teeth, and blood vessels are the major protein constituents of the extracellular matrix and the most abundant proteins in all higher organisms including marine animals. It is an important fraction of marine organic nitrogen, and its degradation is important for marine nitrogen recycling.

Because the tightly coiled triple helical collagen molecule assembles into water-insoluble fibers, collagens are resistant to most proteases except for a limited number of collagenolytic proteases. The mechanism of marine collagen degradation in marine nitrogen recycling is rather unclear because there are only several reports about marine collagenolytic enzyme-producing bacteria and collagenases (1–5). Collagenolytic proteases include metalloproteinases, serine proteases, and other proteases. Of all the collagenolytic proteases, mammalian collagenases, which are mammalian matrix metalloproteinases have been investigated in more detail than collagenolytic proteases from bacteria (6). Studies on the collagenolytic proteases from marine bacteria are rather minor and preliminary. Some marine collagenolytic enzyme-producing bacteria have been reported (1, 5). The collagenase from marine Vibrio B-30 was purified and primarily characterized, which was reported at 1978 and 1980 (2–4). Since then, there has been no report on the characterization of marine bacterial collagenolytic proteases. The lack of knowledge of marine collagenolytic proteases is a huge holdback on the elucidation of the mechanism of marine collagen degradation.

Collagens are water-insoluble fibers and thus a collagen-binding domain occurs in collagenolytic proteases, which has been proven in mammalian matrix metalloproteinases and bacterial collagenases from land (6). Because there is no report on the gene and structure of a marine collagenolytic protease, it is unknown whether marine collagenolytic proteases have collagen-binding domains. Deseasins are a new type of subtilases mainly secreted by bacteria in deep-sea or lake sediment (7). Compared with other subtilases, deseasins are all multidomain enzymes with a polycystic kidney disease (PKD) domain at their C terminus (7). PKD domain has been found in some chitinases (8–10), cellulases (11), proteases (12), and collagenases (13). Except that the PKD domain of the chitinase ChiA from Alteromonas sp. O-7 was reported to have binding ability to chitin (10), the function of the PKD domain of other enzymes...
has not yet been experimentally proven. Genomic sequence analysis of marine *Gramella forsetii* revealed that many exported proteins contain PKD domains (14), indicating that PKD domains might widely lie in the hydrolases of marine heterotrophic bacteria. Therefore, elucidation of the function of the PKD domain is of universal importance.

Deseasin MCP-01, the main extracellular protease of the psychrotolerant bacterium *Pseudoalteromonas* sp. SM9913, from 1855 meter deep-sea sediment, is the type example of deseasins studied because it is the first deseasin that has been purified and characterized. The catalytic characters of MCP-01 with casein as substrate have been studied (7, 15–17). Moreover, it was noticed that the multidomain structure of MCP-01 is similar to that of some hydrolases capable of digesting insoluble biopolymer, such as celluloses (11), chitinases (8–10), and collagenolytic proteases (13), and that the C-terminal PKD domain of MCP-01 looks like a substrate-binding domain just as those in the hydrolases capable of digesting insoluble biopolymer. Thus, it was speculated that deseasin MCP-01 is probably able to hydrolyze insoluble proteins in deep-sea. In this article, deseasin MCP-01 was proven to be able to digest insoluble collagens into soluble peptides and its collagenolytic characters were studied. Moreover, the C-terminal PKD domain of MCP-01 was proven to function as a binding domain during insoluble collagen digestion, and its binding properties, as well as its function in the digestion of insoluble collagen by MCP-01 was studied.

**EXPERIMENTAL PROCEDURES**

*Materials*—Deseasin MCP-01 was purified from *Pseudoalteromonas* sp. SM9913 as previously described (7). Fish collagen was extracted from *Pseudoscaena polyactis* with the method described by Song et al. (18). Collagenase from *Clostridium histolyticum* and insoluble type I collagen fiber (bovine achilles tendon) was purchased from Worthington Biochemical Co., type II and IV collagen from BD Biosciences, acid-dissolved type I collagen (calf skin), elastin (bovine neck ligament), Pz-peptide (4-phenylazobenzyloixycarbonyl-Pro-Leu-Gly-Pro-Arg), and Su-AAA (N-succinyl-Ala-Ala-Ala-p-nitroanilide) were from Sigma, and gelatin from Boston Biomedical Inc. *Escherichia coli* DH5a and pET-22b (+) (Novagen) were used as host and plasmid for the construction of expression vectors, and *E. coli* BL21(DE3) (Transgen) was used as expression host.

**Protein Expression and Purification of the Catalytic Domain of MCP-01**—The genome DNA of *Pseudoalteromonas* sp. SM9913 was prepared with the previous method (7). A DNA fragment coding for the catalytic domain (CD) of MCP-01 (from M1 to G419) was amplified by PCR with the genome DNA of *Pseudoalteromonas* sp. SM9913, *Pfu* polymerase (Fermantas), and two primers (P1, 5′-GCTCATATGAAAACAA-GAGCCCACAACCACCACAA-3′, and P2, 5′-CCGTGGAAGAACACCTGATTGTGATATGTA-3′). The obtained fragment was then ligated into the Ndel-Xhol sites of vector pET-22b (+) (DE3). To analyze the binding ability of the PKD domain to insoluble collagen, the EGFP was used to fuse with the PKD domain. The EGFP gene *egfp* was amplified with an overlapping sequence from vector pEGFP-N1 (Clontech) by PCR. The DNA fragment encoding PKD with the same overlapping sequence was also amplified. The two fragments were overlapped by mutagenesis by overlapping extension PCR (20). The chimeric gene was subcloned into pET-22b (+) for the expression of fusion protein PKD-EGFP. Both recombinant vectors were transformed into *E. coli* BL21(DE3). The recombinant PKD-EGFP were all expressed as C-terminal His<sub>6</sub>-tagged proteins by an inducement of 0.6 mM isopropyl β-D-thiogalactopyranoside at 20 °C for 16 h, and purified with His-Bind metal chelating column.

**Site-directed Mutagenesis of the PKD Domain**—Alignment of PKD domains were performed by Clustal X 1.83 (21). Site-directed mutagenesis was carried out by mutagenesis by overlapping extension-PCR with the vector PKD-EGFP as template. Mutated sites were introduced by the primers with single or double mutation. The mutated genes were subcloned into pET-22b (+) and transformed into *E. coli* BL21(DE3). All mutations were confirmed by enzyme digestion and nucleotide sequencing. Fusion proteins were all expressed and purified with the same condition as PKD-EGFP.

**Protein Determination and Enzyme Assays**—Proteins were assayed by the method of Lowry et al. (22) with bovine serum albumin as standard. The collagenolytic activities of deseasin MCP-01 and its CD against collagen were determined with the method provided by Worthington Biochemical Co. (23). The reaction time was 5 h for fish-insoluble collagen fiber and bovine-insoluble type I collagen fiber, and 0.5 h for bovine-insoluble types I, II, and IV collagen and gelatin. For insoluble collagen, 1 unit equals 1 nmol of l-leucine equivalents released.
Hydrolysis of Insoluble Collagen by Deseasin MCP-01

from collagen in 1 h. For soluble collagens and gelatin, 1 unit equals 1 nmol of l-leucine equivalents from collagen in 1 min. The activity of MCP-01 to Pz-peptide was carried out with the method described by Miyake et al. (24). The enzyme activity to casein and Su-AAA (N-succinyl-Ala-Ala-Ala-p-nitroanilide) was measured with the methods previously described (7). The activity of the collagenease from C. histolyticum to all of the above substrates was measured with the same method as for MCP-01.

To determine the effect of temperature on the activity of MCP-01 and the collagenase from C. histolyticum to bovine-insoluble type I collagen, the enzyme activities were measured from 0 to 80 °C. 50 mM Tris-HCl from pH 6.0 to 9.0 was used to determine the preferable pH profile of MCP-01 in the digestion of bovine-insoluble type I collagen fiber. Various metal ions and inhibitors were added into buffer A to determine their influence on the activity of MCP-01 to bovine-insoluble type I collagen fiber.

**Digestion Pattern Analysis of Insoluble Collagen Fiber by MCP-01**—Fish-insoluble collagen fiber and bovine-insoluble type I collagen fiber were digested by MCP-01 at 60 °C for 5 h, respectively. After centrifugation, the supernatant was assayed with a FP-6500 spectrofluorometer. The activities of MCP-01 and the collagenase from C. histolyticum to various substrates at 40 °C were measured with the methods described under “Experimental Procedures.” The data represent the mean of three experimental repeats with S.D. ≤ 5.

**TABLE 1**
The substrate specificity of desesasin MCP-01 compared with that of the collagenease from C. histolyticum

| Substrate                        | MCP-01 | Collagenase* |
|----------------------------------|--------|--------------|
| Fish-insoluble collagen fiber    | 10434  | 4466         |
| Bovine-insoluble type I collagen | 1608   | 5274         |
| Bovine-acid-dissolved type I collagen | 136.7  | 113.1        |
| Bovine type II collagen          | 67.6   | 157.8        |
| Bovine type IV collagen          | 89.3   | 97.0         |
| Gelatin                          | 151.0  | 181.5        |
| Casein                           | 0.68*  | 0            |
| Pz-PLGPR*                        | 24.8*  | 24.6         |
| Su-AAA*                          | 2.94*  | 0            |

* The collagenease from C. histolyticum purchased from Worthington Biochemical Corp.

The path length of the cuvette was 0.1 cm. The raw CD data were converted into mean residue ellipticity (deg cm$^2$/dmol$^{-1}$) at the entire wavelength using the relation: $[\theta]_\lambda = \theta_\lambda M_0/10/c$.

RESULTS

Characterization of the Collagenolytic Activity of Deseasin MCP-01

Substrate Specificity—Deseasin MCP-01 purified from the culture of Pseudoalteromonas sp. SM9913 was used to hydrolyze some proteins and synthetic peptides to determine its substrate specificity. All the collagens used fish collagen and bovine type I (both insoluble and acid-dissolved), II, and IV collagens, as well as Pz-peptide were all suitable substrates for desesasin MCP-01. For the insoluble collagens from different sources, MCP-01 had higher activity to fish collagen and lower activity to bovine collagen than the collagenease from C. histolyticum. For soluble collagens, the order of preferred substrate for MCP-01 was gelatin > acid-dissolved type I collagen > type IV collagen > type II collagen, and the order for the collagenease from C. histolyticum was gelatin > type II collagen > acid-dissolved type I collagen > type IV collagen. MCP-01 had nearly the same specific activity to Pz-peptide as the collagenease from C. histolyticum. For other proteins, MCP-01 had obvious activity to casein, as well as a slight activity to elastin according to its slight activity to Su-AAA, whereas the collagenease from C. histolyticum had no activity to casein and Su-AAA (Table 1). These results showed that desesasin MCP-01 has broad specificity to various collagens. Moreover, as a marine protease, MCP-01 preferably degrades the collagen from marine animals than that from terrestrial animals. Collagens are the most abundant proteins of marine animals and are an important fraction of marine organic nitrogen. Therefore, it could be suggested that MCP-01 would have high ability to degrade various collagens in deep-sea sediments and therefore play an important role in the recycling of marine nitrogen.

Digestion Pattern of Insoluble Collagen Fiber by MCP-01—The digestion pattern of fish and bovine-insoluble collagen fiber by MCP-01 was analyzed by SDS-PAGE. After a 5-h digestion by MCP-01 at 60 °C, all fish-insoluble collagen fiber in the tube disappeared, whereas those in the control tubes without MCP-01 had only a little decrease. No peptide could be detected from the digestion solution of fish collagen by 12.5% SDS-PAGE (Fig. 1A), showing that MCP-01 decomposed fish-insoluble collagen fiber completely into amino acids and small peptides lower than 10 kDa. As for bovine type I collagen fiber, after a 5-h digestion, almost all the collagen fiber in the tube with MCP-01 disappeared, whereas the fiber in the control tube without MCP-01 had no visible change. SDS-PAGE analysis

| Substrate                        | MCP-01 | Collagenase* |
|----------------------------------|--------|--------------|
| Fish-insoluble collagen fiber    | 10434  | 4466         |
| Bovine-insoluble type I collagen | 1608   | 5274         |
| Bovine-acid-dissolved type I collagen | 136.7  | 113.1        |
| Bovine type II collagen          | 67.6   | 157.8        |
| Bovine type IV collagen          | 89.3   | 97.0         |
| Gelatin                          | 151.0  | 181.5        |
| Casein                           | 0.68*  | 0            |
| Pz-PLGPR*                        | 24.8*  | 24.6         |
| Su-AAA*                          | 2.94*  | 0            |
showed that a large amount of soluble peptides with molecular mass lower than 100 kDa were released from bovine collagen digestion by MCP-01 (Fig. 1A). These results further showed that MCP-01, as a marine collagenolytic protease, preferably decomposed fish collagen more than bovine collagen. Because the sequences of chain α1 and chain α2 of bovine type I collagen were clear, we tried to determine the cleavage sites of MCP-01 on them. The peptides released from bovine collagen were separated in SDS-PAGE and submitted to N-terminal sequence analysis. Finally, the N-terminal sequences of 6 released peptides in the peptide bands in SDS-PAGE were determined (Fig. 1B). A time course analysis of the digestion pattern of bovine type I collagen fiber by MCP-01 showed that the peptide with the N-terminal sequence of SGLDG and molecular mass of about 58 kDa was preferentially derived during the digestion (Fig. 1B). According to the determined N-terminal sequences, 37 possible cleavage sites of MCP-01 on type I collagen chain α1 and chain α2 were deduced (Fig. 2), suggesting that MCP-01 had various but specific cleavage sites on type I collagen fiber. This digestion pattern was similar to that of other bacterial collagenolytic enzymes (28), but different from that of the mammalian collagenses. Mammalian collagenses only had one specific cleavage site on collagen to produce three-quarter and one-quarter fragments (29).

Effects of Temperature and pH on the Collagenolytic Activity of MCP-01—With bovine-insoluble type I collagen fiber as substrate, MCP-01 was most active at 60 °C, and kept 12.4% of the highest activity at 0 °C. In contrast, the collagenase from C. histolyticum was most active at 50 °C, and almost no activity at 0 °C could be detected (Fig. 3A). This showed that, as a cold-adapted enzyme from a deep-sea psychrotolerant bacterium, MCP-01 has high activity at low temperature to be adapted to the cold environment. Cold-adapted enzymes usually have an optimum temperature lower than 40 °C because of their thermolability. Cold-adapted MCP-01 had an unusual high optimum temperature of 60 °C in the digestion of insoluble type I collagen fiber. Because type I collagen can be converted to gelatin at 60 °C and MCP-01 had high gelatinolytic activity (Table 1), most of the activity of MCP-01 at 60 °C might result from its gelatinolytic activity.

With bovine-insoluble type I collagen fiber as substrate in Tris-HCl buffer, deseasin MCP-01 had an optimum at pH 9.0, and over 60% activity remained between pH (35-9)
Hydrolysis of Insoluble Collagen by Deseasin MCP-01

6.0 and 10.0 (Fig. 3B). Our experimental results showed MCP-01 had no collagenolytic activity in acetate, phosphate, carbonate, and a broad pH buffer containing sodium citrate. Therefore, the activity of MCP-01 to type I collagen fiber below pH 6.0 and over pH 10.0 could not be carried out.

Effects of Metal Ions and Protease Inhibitors on the Collagenolytic Activity of MCP-01—Effects of 13 metal ions on the activity of deseasin MCP-01 to insoluble type I collagen fiber were measured. Among these metal ions, only Ca\(^{2+}\) (4 mM) markedly increased the enzyme activity by 174.1%. Zn\(^{2+}\), Ni\(^{2+}\), Cu\(^{2+}\), and Fe\(^{2+}\) severely inhibited the enzyme activity by more than 50%. Co\(^{2+}\), Mg\(^{2+}\), Ba\(^{2+}\), Sn\(^{2+}\), and Mn\(^{2+}\) moderately inhibited the enzyme activity by 38.7, 20.1, 17.4, 11.1, and 10.7% at 4 mM, respectively. Sr\(^{2+}\) and Li\(^{+}\) had a slight inhibitory effect and K\(^{+}\) had no effect on the enzyme activity. As a serine protease, the activity of MCP-01 to type I collagen fiber was conspicuously inhibited by the serine protease inhibitor phenylmethylsulfonyl fluoride (96.1% inhibition, 5 mM). In addition, it was also inhibited by the divalent cation chelators EDTA (92.1%, 1 mM) and EGTA (73.6%, 1 mM), \(\alpha\)-phenanthroline (36.6%, 5 mM), and sodium citrate (71.2%, 5 mM) probably because the Ca\(^{2+}\) in the catalytic domain of MCP-01 was deprived by these chelators (Table 2). These results showed that some metal ions and inhibitors had different and significant effects on the collagenolytic activity of MCP-01, which should be considered in the study and application of MCP-01.

Collagen-binding Properties of the PKD Domain of MCP-01—The PKD domain were expressed in E. coli as an EGFP fusion protein (PKD-EGFP) and purified with HisBind metal chelating column (Fig. 5). Then its binding ability to the insoluble collagen fiber was assayed by a spectrofluorometer with EGFP.
as control. As shown in Fig. 5, PKD-EGFP displayed a significant binding ability to the insoluble collagen fiber, whereas EGFP had no binding ability, showing that the PKD domain of MCP-01 has binding ability to the insoluble collagen fiber. Therefore, the PKD domain of MCP-01 functions as a binding domain in the insoluble collagen fiber digestion by MCP-01. Besides, the PKD domain of MCP-01 had no binding ability to insoluble elastin, which may be another important component of deep-sea sedimentary particulate organic nitrogen (PON) (Fig. 5).

**Determination of the Key Residue in PKD Domain for Collagen Binding**—To clarify the important amino acid residues in PKD domain for collagen binding, side-directed mutagenesis was introduced. The PKD domain sequences of MCP-01, a chitinase from *Alteromonas* sp. O-7 (10), and a collagen-binding region of a collagenolytic protease from *Geobacillus collag-

**FIGURE 5.** A, binding ability of PKD domain and its mutants to bovine-insoluble type I collagen fiber and elastin. B, purified EGFP and fusion proteins of PKD and its three mutants. Various concentrations of EGFP, and fusion proteins of PKD and its mutants were incubated with a fixed amount of collagen (or elastin) in double distilled water at 40 °C for 1 h with mixing. The free fluorescence intensity in the solution before and after incubation was determined with spectrophuorometer. EGFP was used as the control. As shown in Fig. 5, PKD-EGFP displayed a significant binding ability to the insoluble collagen fiber, whereas EGFP had no binding ability, showing that the PKD domain of MCP-01 has binding ability to the insoluble collagen fiber. Therefore, the PKD domain of MCP-01 functions as a binding domain in the insoluble collagen fiber digestion by MCP-01. Besides, the PKD domain of MCP-01 had no binding ability to insoluble elastin, which may be another important component of deep-sea sedimentary particulate organic nitrogen (PON) (Fig. 5).

**FIGURE 4.** A, effect of exogenous PKD domain on the digestion of bovine-insoluble type I collagen fiber by the CD of MCP-01. B, the purified recombinant CD. C, the purified recombinant PKD. Insoluble type I collagen fiber (5 mg) was first incubated in the absence and presence of PKD (1 or 5 molar eq) in 50 mM Tris-HCl (with 0.36 mM CaCl₂, pH 7.5) for 5 h at 40 °C, and then equivalents (200 pmol) of MCP-01 (first column) and CD (second column) were added, respectively, and incubated for another 5 h for activity assay. Enzyme activity was determined as mentioned under “Experimental Procedures.”

**FIGURE 5.** A, effect of exogenous PKD domain on the digestion of bovine-insoluble type I collagen fiber by the CD of MCP-01. B, the purified recombinant CD. C, the purified recombinant PKD. Insoluble type I collagen fiber (5 mg) was first incubated in the absence and presence of PKD (1 or 5 molar eq) in 50 mM Tris-HCl (with 0.36 mM CaCl₂, pH 7.5) for 5 h at 40 °C, and then equivalents (200 pmol) of MCP-01 (first column) and CD (second column) were added, respectively, and incubated for another 5 h for activity assay. Enzyme activity was determined as mentioned under “Experimental Procedures.”

**Discussion**

Deseasin MCP-01 is the main extracellular protease of the deep-sea bacterium *Pseudoalteromonas* sp. SM9913 (15). It has been proven to be a cold-adapted multidomain subtilase with high autolytic activity, whose enzymatic characters have been studied with casein as substrate (7, 15–17). In this article, it was proven that deseasin MCP-01 is a collagenolytic serine protease, capable of digesting various collagens from both terrestrial and marine animals. Moreover, as a marine collagenolytic protease, it is more prone to degrade marine collagen than terrestrial collagen. Collagens are the major protein components of the extracellular matrix and the most abundant proteins in all higher organisms including marine animals. Therefore, it is an important fraction of marine organic nitrogen, and its degradation is important for marine nitrogen recycling. The broad specificity of MCP-01 to collagens suggests that MCP-01 would have a high ability to degrade various collagens in deep-sea sediments and therefore play an important role in the recycling of marine nitrogen.

Of all the collagenolytic proteases, mammalian collagenase that belong to matrix metalloproteinases have been investigated in detail. Mammalian collagenases have the same modular structure with an N-terminal domain, a linker peptide, and a C-terminal domain. The N-terminal domain is a catalytic domain belonging to M10, and the C-terminal domain is a collagen-binding domain with a unique four-bladed β-propeller structure (30). Although it is a serine protease, deseasin MCP-01 has the same modular structure as mammalian collagenases, which contains an N-terminal catalytic domain belonging to Ser-8, a linker peptide and a C-terminal PKD domain (7). The PKD domain has similar structure and function as the C-terminal domain of mammalian collagenases. It

**Hydrolysis of Insoluble Collagen by Deseasin MCP-01**

Deseasin MCP-01 is the main extracellular protease of the deep-sea bacterium *Pseudoalteromonas* sp. SM9913 (15). It has been proven to be a cold-adapted multidomain subtilase with high autolytic activity, whose enzymatic characters have been studied with casein as substrate (7, 15–17). In this article, it was proven that deseasin MCP-01 is a collagenolytic serine protease, capable of digesting various collagens from both terrestrial and marine animals. Moreover, as a marine collagenolytic protease, it is more prone to degrade marine collagen than terrestrial collagen. Collagens are the major protein components of the extracellular matrix and the most abundant proteins in all higher organisms including marine animals. Therefore, it is an important fraction of marine organic nitrogen, and its degradation is important for marine nitrogen recycling. The broad specificity of MCP-01 to collagens suggests that MCP-01 would have a high ability to degrade various collagens in deep-sea sediments and therefore play an important role in the recycling of marine nitrogen.

Of all the collagenolytic proteases, mammalian collagenase that belong to matrix metalloproteinases have been investigated in detail. Mammalian collagenases have the same modular structure with an N-terminal domain, a linker peptide, and a C-terminal domain. The N-terminal domain is a catalytic domain belonging to M10, and the C-terminal domain is a collagen-binding domain with a unique four-bladed β-propeller structure (30). Although it is a serine protease, deseasin MCP-01 has the same modular structure as mammalian collagenases, which contains an N-terminal catalytic domain belonging to Ser-8, a linker peptide and a C-terminal PKD domain (7). The PKD domain has similar structure and function as the C-terminal domain of mammalian collagenases. It
Hydrolysis of Insoluble Collagen by Deseasin MCP-01

FIGURE 7. CD spectra of PKD domain and its W36A mutant. The spectra were measured on a Jasco J-810 spectropolarimeter (Japan) with the method described under "Experimental Procedures." The straight line represents PKD and the dotted line represents its W36A mutant.

has a β-sandwich fold with 5 parallel β-sheets (7, 31) and has collagen-binding ability proven in this article. Mammalian collagenases hydrolyze various collagens with significant quantitative differences in activity and specificity. They cleave collagens at a single site to produce characteristic three-quarter and one-quarter fragments (29, 30). Deseasin MCP-01 had broad specificity to various collagens. However, unlike mammalian collagenases, deseasin MCP-01 had various but specific cleavage sites on type I collagen to produce various fragments as shown in Fig. 1B.

Among collagenolytic proteases from bacteria, metalloproteases are the most frequently occurring, whereas the number of serine proteases is rather minor (6). Among collagenolytic serine proteases, the thermostable protease of G. collagenovorans MO-1 isolated from soil is studied in most detail. It is a 210-kDa protease consisting of two identical subunits (25, 28). Deseasin MCP-01 is a cold-adapted protease of 65.84 kDa from the deep-sea sedimentary bacterium Pseudoalteromonas sp. SM9913 (7). The identity of their sequences is only 22.7%. Despite these differences, these two proteases have some similar characteristics on their collagenolysis. Similar to the protease from G. collagenovorans MO-1, MCP-01 exhibited broad substrate specificity to various type collagens. Moreover, MCP-01 had various but specific cleavage sites on insoluble collagen, and thus insoluble collagen fiber was digested into dissolved peptides smaller than 100 kDa. According to the N-terminal sequences of some released peptides, 37 possible cleavage sites of MCP-01 on bovine collagen chains were deduced. Unlike the thermostable protease from G. collagenovorans MO-1, MCP-01 is a cold-adapted enzyme and remained as 12.4% collagenolytic activity at 0 °C. In addition, its collagenolytic activity exhibited an alkaline profile. These reflect the adaptation of the collagenolytic activity of MCP-01 to the cold and alkaline deep-sea environment. MCP-01 had a high optimum temperature of 60 °C, which may result from its high gelatinolytic activity.

It is well known that many enzymes responsible for digestion of insoluble polymers, such as xylanases, cellulases, chitinases, and collagenases, contain substrate-binding domains (6, 10, 32, 33). Collagenolytic metalloproteases from bacteria have been reported to have collagen-binding domains (6). Among collagenolytic serine proteases, only the protease from G. collagenovorans MO-1 has been experimentally proven to have a collagen-binding region (25). MCP-01 and other deseasins all have a C-terminal PKD domain (7). PKD domain widely lies in chitinases (8–10), cellulases (11), collagenases (13), and proteases (12). The PKD domain of chitinase ChiA from Alteromonas sp. O-7 has been proven to have chitin-binding ability (10). However, the function of the PKD domain in other enzymes has not been reported. In this article, the PKD domain of deseasin MCP-01 was proven to function as a binding domain to bind insoluble collagen fiber during its digestion. Not only did it bind insoluble collagen fiber, but it also functioned on collagen fiber to facilitate fiber digestion by MCP-01, a mechanism that needs to be investigated further. Aromatic residues usually play key roles in the binding of binding domains to insoluble substrates (26, 27). Site-directed mutagenesis proved that Trp-36 plays a key role in the binding of the PKD domain of MCP-01 to collagen fiber. To our knowledge, PKD domain was experimentally proven to bind a protein for the first time. Genomic sequence analysis of marine G. forsetii revealed that 14 susCD-like open reading frames frequently encode exported proteins with PKD domains (14), which suggests that PKD domains probably widely lie in the hydrolases of marine heterotrophic bacteria. The result in this article and that in the report by Orikoshi (10) demonstrate that the PKD domains in marine hydrolases function as a binding domain binding not only insoluble organic carbon such as chitin but also insoluble organic nitrogen such as collagen.

Alignment of the amino acid sequence of the PKD domain of deseasin MCP-01 with those from the chinease ChiA and the protease from G. collagenovorans MO-1. ALTCHIA, the PKD domain of chinease ChiA from Alteromonas sp. strain O-7 (AB063629); GEOCOL, the intervening region of the collagenolytic protease from G. collagenovorans MO-1 (AB260948); PKD, the PKD domain of deseasin MCP-01 from Pseudoalteromonas sp. SM9913. Identical residues are indicated by asterisks. Conserved aromatic residues are indicated by bold letters. Sequences were aligned using Clustal X 1.83 (21).

FIGURE 6. Alignment of the amino acid sequence of the PKD domain of deseasin MCP-01 with those from the chinease ChiA and the protease from G. collagenovorans MO-1. ALTCHIA, the PKD domain of chinease ChiA from Alteromonas sp. strain O-7 (AB063629); GEOCOL, the intervening region of the collagenolytic protease from G. collagenovorans MO-1 (AB260948); PKD, the PKD domain of deseasin MCP-01 from Pseudoalteromonas sp. SM9913. Identical residues are indicated by asterisks. Conserved aromatic residues are indicated by bold letters. Sequences were aligned using Clustal X 1.83 (21).
Therefore, they are important for the digestion of marine insoluble biopolymers.

There is yet no gene or structure of a marine collagenolytic protease elucidated, and consequently the digestion mechanism of marine animal collagens by collagenases is unclear. This article demonstrated that the marine collagenolytic protease MCP-01 has similar structural architecture to those from the land, having a collagen-binding domain, and a similar mechanism for collagen digestion, binding insoluble collagens by its binding domain, and then degrading them into soluble peptides and amino acids.

The total input of PON from seawater to the deep-sea sediment is \( 36 \pm 21 \, \mu \text{mol} \, \text{m}^{-2} \, \text{d}^{-1} \) (34). Considering the vast area of deep-sea floor and such a huge input of PON, the recycling of PON in deep-sea sediment would be a non-negligible part of global nitrogen cycling. However, it is still unclear how these PON were degraded because the identification of the microorganisms and the enzymes participating in this process are scarce (34, 35). Because MCP-01 has specific activity to insoluble protein, MCP-01 would play an important role in the degradation of deep-sea sedimentary PON. Besides MCP-01, most desesains are from bacteria in deep-sea or lake sediment. Therefore, desesains and their original bacteria may play an important role in the decomposition of sedimentary PON, which still needs further confirmation by experiments on other desesains.

REFERENCES

1. Merkel, J. R., Dreisbach, J. H., and Ziegler, H. B. (1975) *Appl. Microbiol.* **29**, 145–151

2. Dreisbach, J. H., and Merkel, J. R. (1978) *J. Bacteriol.* **135**, 521–527

3. Merkel, J. R., and Dreisbach, J. H. (1978) *Biochemistry* **17**, 2857–2863

Worthington Enzyme Manual, 27, 2951–2961

7. Worthington Biochemical Corp. (1972)

10. An, Y. F., and Ji, J. F. (2005) *Appl. Microbiol. Biotechnol.* **68**, 774–777

13. Matsushita, O., Jung, C. M., Katayama, S., Minami, J., Takahashi, Y., and Okabe, A. (1999) *J. Bacteriol.* **181**, 923–936

16. Chen, X. L., Sun, C. Y., Zhang, Y. Z., and Gao, P. J. (2003) *Biotecnol. Lett.* **25**, 1763–1767

19. Laemmli, U. K. (1970) *Nature* **227**, 680–685

22. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) *Nucleic Acids Res.* **25**, 4676–4882

25. Miyake, R., Shigeri, Y., Tatsu, Y., Yumoto, N., Umekawa, M., Tsujimoto, Y., Matsui, H., and Watanabe, K. (2005) *J. Bacteriol.* **187**, 4140–4148

28. Nagy, T., Simpson, P., Williamson, M. P., Hazlewood, G. P., Gilbert, H. J., and Orosz, L. (1998) *FEBS Lett.* **429**, 312–316

31. Bycroft, M., Bateman, A., Clarke, J., Hamill, S. J., Sandford, R., Thomas, R. L., and Chorthia, C. (1999) *EMBO J.* **18**, 297–305

34. Brunngräd, J., Grandell, S., Stahl, H., Tengberg, A., and Hall, P. O. J. (2004) *Prog. Oceanogr.* **63**, 159–181

37. Jorgensen, B. B., and Boetius, A. (2007) *Nature* **5**, 770–781