Mechanistic Insight into the Binding and Swelling Functions of Prepeptidase C-Terminal (PPC) Domains from Various Bacterial Proteases

JiaFeng Huang,^a RiBang Wu,^a Dan Liu,^a BinQiang Liao,^a Ming Lei,^a Meng Wang,^a Ran Huan,^a MingYang Zhou,^b ChangBei Ma,^a HaiLun He^a

^aSchool of Life Sciences, Central South University, Changsha, People's Republic of China

^bShandong Provincial Key Laboratory of Fine Chemicals, School of Chemistry and Pharmaceutical Engineering, Qilu University of Technology (Shandong Academy of Sciences), Jinan, People's Republic of China

ABSTRACT The bacterial prepeptidase C-terminal (PPC) domain can be found in the C termini of a wide variety of proteases that are secreted by marine bacteria. However, the functions of these PPC domains remain unknown due to a lack of systematic research. Here, the binding and swelling abilities of eight PPC domains from six different proteases were compared systematically via scanning electron microscopy (SEM), enzyme assays, and fluorescence spectroscopy. These PPC domains all possess the ability to bind and swell insoluble collagen. PPC domains can expose collagen monomers but cannot disrupt the pyridinoline cross-links or unwind the collagen triple helix. This ability can play a synergistic role alongside collagenase in collagen hydrolysis. Site-directed mutagenesis of the PPC domain from *Vibrio anguillarum* showed that the conserved polar and aromatic residues Y6, D26, D28, Y30, W42, E53, C55, and Y65 and the hydrophobic residues V10, V18, and I57 played key roles in substrate binding. Molecular dynamic simulations were conducted to investigate the interactions between PPC domains and collagen. Most PPC domains have a similar mechanism for binding collagen, and the hydrophobic binding pocket of PPC domains may play an important role in collagen binding. This study sheds light on the substrate binding mechanisms of PPC domains and reveals a new function for the PPC domains of bacterial proteases in substrate degradation.

IMPORTANCE Prepeptidase C-terminal (PPC) domains commonly exist in the C termini of marine bacterial proteases. Reports examining PPC have been limited, and its functions remain unclear. In this study, eight PPCs from six different bacteria were examined. Most of the PPCs possessed the ability to bind collagen, feathers, and chitin, and all PPCs could significantly swell insoluble collagen. PPCs can expose collagen monomers but cannot disrupt pyridinoline cross-links or unwind the collagen triple helix. This swelling ability may also play synergistic roles in collagen hydrolysis. Comparative structural analyses and the examination of PPC mutants revealed that the hydrophobic binding pockets of PPCs may play important roles in collagen binding. This study provides new insights into the functions and ecological significance of PPCs, and the molecular mechanism of the collagen binding of PPCs was clarified, which is beneficial for the protein engineering of highly active PPCs and collagenase in the pharmaceutical industry and of artificial biological materials.

KEYWORDS collagen, collagen swelling, mechanism, mutagenesis, prepeptidase C-terminal domain, PPC domain, substrate binding diversity

Marine bacteria play critical roles in biogeochemical cycling in seawater ecosystems (1, 2). Particular organic nitrogen (PON) refers to high-molecular-weight dissolved organic nitrogen sources in the environment (1). Insoluble collagen and elastin are

Citation Huang J, Wu R, Liu D, Liao B, Lei M, Wang M, Huan R, Zhou M, Ma C, He H. 2019. Mechanistic insight into the binding and swelling functions of prepeptidase C-terminal (PPC) domains from various bacterial proteases. *Appl Environ Microbiol* 85:e00611-19. <https://doi.org/10.1128/AEM.00611-19>.

Editor Haruyuki Atomi, Kyoto University

Copyright © 2019 American Society for Microbiology. All Rights Reserved.

Address correspondence to HaiLun He, helenhe@csu.edu.cn.

J.H. and R.W. contributed equally to this work.

Received 14 March 2019

Accepted 29 April 2019

Accepted manuscript posted online 10 May 2019

Published 1 July 2019

abundant in marine animals and represent major sources of marine PON (3, 4). Insoluble collagen and elastin in marine animals can be degraded into dissolved organic nitrogen by microorganisms, such as the M12 protease myroilysin from the marine bacterium *Myroides profundus* D25 (1, 5) and the serine protease MCP-01 from the deep-sea bacterium *Pseudoalteromonas* sp. strain SM9913 (6). Many pathogenic bacteria require the secretion of proteases that act as pathogenic factors that damage collagenous fibrils and expose subcutaneous tissues and vessels (7, 8). The metalloprotease VvpE from *Vibrio vulnificus* can cause the breakdown of fibrin and interfere with blood homeostasis (9). Although the sequences and spatial structures of bacterial collagenolytic or elastinolytic proteases vary among species, many proteases contain C-terminal domains, such as the polycystic kidney disease (PKD) domain (10), the P-protein (P) domain (11), the prepeptidase C-terminal (PPC) domain (3, 12), and the collagen-binding domain (CBD) (13, 14). These domains have different sequences, structures, and functions. Sequence alignment shows that there is no homology among these four domains (<15%) (data not shown). Despite the fact that these domains are rich in β -sheets, the structures of these C-terminal domains are also quite different: the CBD is a type of single-stranded, left-handed beta-helix domain, the P domain is a galactose-binding domain-like domain, and the PKD and PPC domains are immunoglobulin-like (Ig-like) beta-sandwich domains. Many researchers have shown that C-terminal domains can have significant impacts on the structures and functions of proteases (1, 3, 10, 15). In our previous systematic review, we summarized the functions of the C-terminal domains of bacterial proteases, including the regulation of the secretory process, the anchoring and swelling of substrate molecules, the inhibition of preproteases, and the maintenance of structural stability/flexibility (12).

The bacterial PPC domain, which contains 70 to 90 amino acids, is commonly found at the C termini of secreted peptidases in bacteria and archaea, especially in marine and pathogenic bacteria (3, 12). The PPC domain has no hydrolytic activity; however, the PPC domain is crucial for the recognition and turnover of certain substrates. An active enzyme may include one catalytic domain combined with one or two PPC domains (3). Some studies have shown that the PPC domain plays a role in substrate hydrolysis (16–18). Our previous studies also found that the PPC domain from the metalloprotease E495 had the ability to bind C-phycocyanin and casein, which could efficiently facilitate the hydrolysis of the catalytic domain (3). However, there remains a lack of comprehensive and systematic studies examining the functional diversity of PPC domains from various bacteria and their roles in environmental adaptation. The PPC domain is a typical Ig-like beta-sandwich domain (19). In addition, structural alignment showed that PPC-like structures exist not only in bacteria and archaea but also in eukaryotes, such as the C2-like domain (20), the gold domain (21), and the ephrin type A receptor 4 domain (22). Despite their low sequence identities, PPC-like protein domains share similar secondary and tertiary structures (see Fig. S1 in the supplemental material). The similarity among the structures of PPC-like domains indicates that PPC domains may perform similar functions (20–22). Research examining PPC domains would not only be helpful for determining the physiological significance of PPC domains in marine microorganisms but would also be beneficial for understanding the functions of PPC-like structures.

In this paper, we compared eight PPC domains from four marine bacteria and one halotolerant bacterium and showed that the PPC domains not only had substrate-binding abilities but were also able to swell collagen. Furthermore, scanning electron microscopy (SEM), fluorescence spectra, and enzyme assays were used to detect the functions of the PPC domains in collagenolysis and the effects of PPC domains on collagen structures. Moreover, the residues within the EmpA-PPC domain that are necessary for protein binding and collagen swelling were determined by site-directed mutagenesis.

FIG 1 Network analyses of the evolutionary relationships among PPC domains based on the evolutionary distance among PPC domains. Each node represents a bacterium. The strains are connected with each other according to their evolutionary distance. The node size is proportional to the weight of the links connected to each node. Evolutionary distances lower than 0.2 are presented. The network was generated using Gephi software.

RESULTS AND DISCUSSION

Diversity of PPC domains and phylogenetic analysis. According to the EMBL-EBI Pfam database, the bacterial prepeptidase C-terminal domain (PF04151) is classified as a PPC domain. In addition, the PPC domains that are documented in the Pfam database are also annotated as PPC domains in the MEROPS and NCBI Conserved Protein (identifier [ID] 309326) databases. Although the PPC domain is called “peptidase, C-terminal, archaeal/bacterial” and is classified as IPR007280 in the InterPro database, the descriptions of PPC domains in these data banks are similar. PPC domains are commonly found at the C termini of bacterial and archaeal proteases, the majority of which belong to the MEROPS M4, M9, and M28 families of peptidase metalloproteases and to the S8 family of serine proteases (3, 12).

As shown in Fig. 1, PPC domains can be found in at least 34 bacterial genera, including *Aeromonas*, *Rhodanobacter*, *Luteibacter*, *Colwellia*, *Kangiella*, *Stenotrophomonas*, *Teredinibacter*, *Shewanella*, *Vibrio*, and *Pseudoalteromonas*. PPC domains from the same bacterial genera cluster together, while PPC domains from different species, such

TABLE 1 GST-PPC recombinant proteins used in this study

Recombinant protein	PPC no.	Mol wt (Da)	Protease with PPC	UniProt identifier	GenBank accession	Source strain
EmpA-PPC	1	36,384	Metalloprotease EmpA	Q6T863	AAR88093.1	<i>V. anguillarum</i>
VVP-PPC	1	35,032	Metalloprotease VVP	Q06AK2	ABI95803	<i>V. vulnificus</i>
YHS-PPC	1	35,692	Serine protease YHS	A0A3R5SNZ0	QAB01366.1	<i>Salinivibrio</i> sp. YH4
YHM-PPC	1	35,146	Metalloprotease YHM	A0A3R5UTQ7	QAB01367.1	<i>Salinivibrio</i> sp. YH4
E423-PPC1	1	35,003	Metalloprotease E423	A0A1I9RW76	AOZ35395	<i>Pseudoalteromonas</i> sp. CSN423
E423-PPC2	1	35,226	Metalloprotease E423			<i>Pseudoalteromonas</i> sp. CSN423
E423-PPC12	2	46,073	Metalloprotease E423			<i>Pseudoalteromonas</i> sp. CSN423
J2-PPC1	1	35,245	Metalloprotease J2	A0A410GIH8	QAB01365.1	<i>Pseudoalteromonas</i> sp. J2
J2-PPC2	1	34,879	Metalloprotease J2			<i>Pseudoalteromonas</i> sp. J2
J2-PPC12	2	46,624	Metalloprotease J2			<i>Pseudoalteromonas</i> sp. J2

as *Vibrio*, *Shewanella*, *Pseudoalteromonas*, *Teredinibacter*, and *Stenotrophomonas*, have long evolutionary distances (Fig. 1). Intriguingly, PPC domains found at different positions of the same proteases can have significantly long evolutionary distances, especially PPC1/PPC2 from *Pseudoalteromonas* and PPC/PPC1/PPC2 from *Teredinibacter* (Fig. 1). Although the sequence similarities among PPC domains are low, there are some highly conserved amino acid residues in the PPC domain, including completely conserved regions and relatively conserved regions (>75%). An analysis based on sequence alignment showed that GG*GDADLYV* and CRPY**GN*E*C were relatively conserved regions within the PPC domain, and these regions may play important roles in maintaining structural stability or function (see Fig. S2 in the supplemental material). Moreover, despite low sequence identities, the secondary structures of different PPC domains from different bacteria are very similar (23) (Fig. S1). Almost all PPC domains consist of β -sheets, and PPC domains are Ig-like β -sandwich domains, indicating that the structures of PPC domains are relatively conserved. The wide distribution and structural conservation of bacterial PPC domains indicate that PPC domains may have similar ecological functions. However, the physiological functions of PPC domains remain unclear.

Functional analysis of different PPC domains. The PPC domain is most abundant in *Pseudoalteromonas* and *Vibrio* (see Fig. S3). Bacteria from these two genera are commonly distributed in the marine environment (4, 23, 24) and represent the primary cultivable bacteria with potential applications. We chose PPC domains from *Pseudoalteromonas* and *Vibrio* as representative PPC domains for analysis of the physiological functions of PPC domains. An alignment of the amino acid sequences of EmpA, VVP, E423, J2, YHM, and YHS, showed that each sequence contained a PPC domain at the C terminus. We constructed 10 glutathione S-transferase (GST)-PPC recombinant fusion proteins, purified the recombinant PPC domains using GST affinity columns, and confirmed each purified fusion protein using SDS-PAGE (Table 1; see also Fig. S4).

(i) Comparisons of the combined functions of PPC domains to bind insoluble substrates. The binding abilities of GST-PPC fusion proteins to bind insoluble collagen, feather meal, and chitin were determined by analyzing the amounts of PPC domain-bound protein segments after incubation with various insoluble substrates. As shown in Fig. 2, YHM-PPC and J2-PPC1 could not bind collagen and E423-PPC1 could not bind chitin, whereas the other PPC domains were able to bind collagen, feather meal, and chitin. The PPC domains from *Vibrio* species (VVP-PPC and EmpA-PPC) appeared to have strong abilities to bind collagen, feather meal, and chitin. Among them, VVP-PPC had a strong collagen-binding ability (51.0%), and EmpA-PPC and E423-PPC1 had strong feather-binding abilities (68.0% and 84.3%, respectively). In addition, YHM-PPC and J2-PPC2 had strong chitin-binding abilities (78.5% and 76.8%, respectively) (see Table S1). E423-PPC12 was able to bind to collagen, feather meal, and chitin, whereas E423-PPC1 only bound to feather meal and bound weakly to collagen; E423-PPC2 bound weakly to collagen, feather meal, and chitin. Similarly, J2-PPC12 also bound to collagen, feather meal, and chitin, while J2-PPC1 only bound weakly to collagen.

FIG 2 The binding abilities of recombinant GST-PPC fusion proteins for insoluble type I collagen, feather meal, and chitin. Substrate (1 mg) was added to 100 μ l PBS (20 mM, pH 7.4) with 0.15 μ M recombinant proteins and incubated at 37°C for 2 h with gentle agitation. After incubation, the supernatant was analyzed by SDS-PAGE. Recombinant proteins without any substrate were used as controls. GST is shown as the internal control. The bar graphs represent the relative values of the recombinant GST-PPC fusion proteins. The bands were quantitated using ImageJ 1.46. Values are expressed as the means $\pm$ SDs ($n = 3$).

(ii) Comparisons of the combined functions of PPC domains to bind soluble collagen and soluble cellulose. An 8-anilino-1-naphthalenesulfonic acid (ANS) fluorescence detection assay was used to detect whether the PPC domains were able to bind soluble collagen and carboxymethylcellulose (25). ANS is an extrinsic negatively charged fluorescent probe, known to bind to hydrophobic patches on protein surfaces. If the PPC domain bound to biomacromolecules, the hydrophobic regions of the PPC domain would be covered, blocking the binding sites for the ANS fluorescence probe (25). As shown in Fig. 3, ANS fluorescence intensity was high only when PPC domains and ANS were included in the reaction system. When PPC domains were preincubated with soluble collagen, the ANS fluorescence intensity was reduced. The results showed that all of the recombinant PPC domains were able to bind strongly to soluble collagen. YHS-PPC, J2-PPC2, EmpA-PPC, and E423-PPC12 had the strongest soluble collagen-binding abilities (98.1%, 92.5%, 85.9%, and 84.8%, respectively), whereas the collagen-binding ability of YHM-PPC was relatively weak. PPC domains bound more strongly with soluble collagen than with insoluble collagen. YHM-PPC and J2-PPC2 were unable to bind insoluble collagen, as shown in Fig. 2; however, both domains still had some collagen-binding ability. Compared with the collagen-binding abilities, the carboxymethylcellulose-binding abilities of the examined PPC domains were relatively weak. PPC domains from the halotolerant bacterium *Salinivibrio* sp. strain YH4 could not bind to cellulose, whereas E423-PPC12, J2-PPC12, EmpA-PPC, and VVP-PPC had low cellulose-binding abilities

FIG 3 The binding abilities of recombinant GST-PPC fusion proteins for soluble collagen and cellulose. (A) ANS fluorescence intensities of soluble collagen and soluble carboxymethylcellulose. (B to K) ANS fluorescence intensities for PPC domains alone (1) and in the presence of collagen (2) or cellulose (3). Values are expressed as the means $\pm$ SDs ($n = 3$).

(Fig. 3; see also Table S2). However, according to the electrophoretic mobility shift assay (EMSA) results (see Fig. S5), the PPC domains did not have the ability to bind linear DNA, likely due to the negative charge of DNA.

Collagen, chitin, keratin, and cellulose are widely distributed in the marine environment. PPC domains were first reported to possess chitin- and keratin-binding abilities. Collagen and keratin represent important components of marine PON (15, 26). Chitin has the ability to adsorb small particulate organic matter in nature and can act as an adsorption matrix for marine organic nitrogen, especially in oligotrophic marine environments (27, 28). Proteases with PPC domains can effectively bind to these substrates, facilitating the hydrolysis of PON by bacterial proteases. The existence of PPC domains could promote the binding of proteases to substrates, which would be beneficial for microorganisms growing in a marine oligotrophic environment and accelerate the marine nitrogen cycle.

(iii) Collagen-swelling abilities of PPC domains. Wang et al. reported the collagen-swelling ability of the PKD domain (10). Similar to PKD, our study showed that PPC domains could also swell insoluble collagen. After the incubation of recombinant GST-PPC fusion proteins with insoluble type I collagen at 37°C for 12 h, the insoluble collagen was obviously swollen; however, tube observation directly showed that GST protein alone could not swell type I collagen (Fig. 4). Compared with 6 M urea, a well-known collagen-swelling agent (1, 29), the collagen-swelling abilities of PPC domains (0.15 μ M) were much stronger, as assessed by measuring changes in collagen volumes (Fig. 4A). We measured the changes in collagen volumes after incubating

FIG 4 The collagen-swelling effects of the PPC domains. (A) Insoluble type I collagen was swollen by different GST-PPC fusion proteins or urea at 37°C for 12 h. A total of 5 mg insoluble collagen in 2 ml 20 mM PBS buffer (pH 7.4) was used as a control. (B) Statistical analysis of the relative collagen volumes of untreated and PPC-treated collagen fascicles. The bands were quantitated using ImageJ 1.46. Values are expressed as the means $\pm$ SDs by taking the average from three measurements.

collagen with PPC domains, and the average volume of PPC-treated collagen increased by approximately 5-fold (Fig. 4B). Viewed macroscopically, EmpA-PPC, E423-PPC12, and J2-PPC12 had stronger swelling effects on type I collagen, with collagen volumes increasing more than 6.5-fold (Fig. 4B). Moreover, two tandem PPC domains (e.g., E423-PPC12 and J2-PPC12) had greater collagen-swelling effects than either individual PPC domain. In addition, when EmpA-PPC was incubated with insoluble collagen at 4°C, 10°C, 20°C, and 30°C, the collagen-swelling effects of EmpA-PPC varied. Although the PPC domain demonstrated swelling abilities at lower temperatures, its collagen-swelling ability increased as temperatures increased from 10°C to 30°C (Fig. 5).

(iv) Effects of PPC domains on collagenase activity. To study whether PPC-treated collagen could more easily be degraded by collagenase, we compared the collagenolytic activities of collagenase when using either PPC-treated or untreated insoluble collagen fibers as the substrates. The results showed that certain PPC domains were able to facilitate insoluble collagen fiber digestion (Fig. 6, Table S2). The collagen samples treated with YHM-PPC, YHS-PPC, E423-PPC1, -PPC2, and -PPC12, and J2-PPC1 and -PPC12 were more easily hydrolyzed by collagenase than untreated collagen. After treatment with J2-PPC1, the collagen fiber degradation rate was increased by approximately 2.4-fold. After treatment with YHM-PPC, YHS-PPC, E423-PPC1 and E423-PPC12, the rates of collagen hydrolysis increased more than 1.5-fold (Fig. 6). The results shown

FIG 5 Collagen swollen by the GST-EmpA-PPC domain for 5 h at different temperatures. Insoluble type I collagen was incubated with 0.15 μ M GST-EmpA-PPC at 4°C, 10°C, 20°C, and 30°C for 5 h each.

FIG 6 Activities of collagenase against untreated collagen fiber, GST-treated collagen, and collagen treated with different GST-PPC fusion proteins. Collagenase activity was assessed by the method described by Lowry et al. (43). Values are expressed as the means $\pm$ SDs ($n = 3$). *, $P < 0.05$. **, $P < 0.01$.

in Table S2 show that some PPC domains can play synergistic roles alongside collagenase in collagen hydrolysis. In the next section, we demonstrate that PPC domains do not unwind the collagen triple helix or break cross-links (see Fig. 9). However, PPC domains can swell collagen and expose collagen monomers, which are more easily recognized by collagenase, an enzyme that hydrolyzes collagen by binding and degrading the collagen triple helix (5, 30, 31). Intriguingly, EmpA-PPC, VVP-PPC, and J2-PPC2 were only able to swell collagen (Fig. 4) and did not appear to facilitate collagen digestion (Fig. 6). As shown in Fig. 4, the collagen-swelling abilities of VVP-PPC and J2-PPC2 were relatively weak. SEM observation of collagen (Fig. 7; see also Fig S6) showed that the collagen fibers released by VVP-PPC and J2-PPC2 were quite thick. EmpA-PPC displayed a strong swelling effect on type I collagen. However, the diameters of the collagen fibers released by EmpA-PPC were still larger than 98 nm (Fig. 7). The abilities of EmpA-PPC, VVP-PPC, and J2-PPC2 to swell collagen without assisting with collagenolysis might be attributed to the reduced exposure of collagen monomers in collagen treated with these three PPC domains.

Collagen is an important component of marine PON that is widely distributed among the skin, scales, bones, tendons, and blood vessels of marine animals, and its degradation is important for marine nitrogen recycling (15). However, the architecture of collagen is notably resistant to hydrolysis by proteases. Many marine bacteria have evolved anchoring units in the C termini of proteases, such as CBD and PKD domains, to assist with enzymatic hydrolysis (7, 15). The CBD domain in collagenase is the minimal segment required for binding to a collagen fibril (15). Similarly, the PKD domain of the metalloprotease MCP-01 is also able to bind insoluble collagen and facilitate collagen degradation (7, 30). In the present study, we studied the functions of PPC domains, such as the abilities of PPC domains to bind, swell, and assist with the hydrolysis of collagen. PPC domains could play a synergistic role in collagen hydrolysis to accelerate the marine nitrogen cycle and could be beneficial for marine bacteria growing in a marine oligotrophic environment. Intriguingly, our study found that combining two PPC domains (E423-PPC12 and J2-PPC12) enhanced collagen-swelling abilities compared with those of either of the individual PPC domains (Fig. 4), suggesting that tandem C-terminal PPC domains may have enhanced functional effects. As far as we know, there have been no reports on the functional promotional effects of multi-PPC domains; however, enhanced binding abilities have been observed for other C-terminal domains. Caviness et al. reported that tandem CBD domains bound more tightly with collagen fibrils than the sum of the individual CBD1 and CBD2 binding abilities (32). In addition, Sekiguchi et al. studied the collagen-binding affinities of the CBD domain for the collagenases ColG and ColH and found that two CBDs had the highest affinity for collagenous peptide (33). These results indicated that multi-CBDs enhanced the collagen-binding abilities of CBD-containing proteins.

FIG 7 SEM observation of collagen fascicles swollen by the PPC domains. Insoluble type I collagen (5 mg) was incubated with 0.15 μ M GST-EmpA-PPC (D, E, and F), GST-E423-PPC12 (G, H, and I), and GST-YHM-PPC (J, K, and L) in 2 ml PBS (20 mM, pH 7.4) at 37°C with continuous mixing for 5 h. The reaction performed with PBS alone (A, B, and C) served as a control. Yellow arrows indicate the diameters of the collagen fibers. Red arrows indicate vesicular projections with diameters of approximately 15 to 30 nm, which are suspected to be inflated collagen fibrils.

Effect of PPC domains on collagen structure. To study the collagen-swelling mechanisms of PPC domains, changes in the structure of collagen fibers caused by PPC domains were observed by SEM. SEM observation suggested that after incubation with GST-EmpA-PPC (Fig. 7D to F), GST-E423-PPC12 (Fig. 7G to I), GST-YHM-PPC (Fig. 7J to L), GST-J2-PPC12, GST-VVP-PPC, and GST-YHS-PPC (Fig. S6) at 37°C for 12 h, the compacted collagen structure became loose, while untreated collagen (Fig. 7A to C) maintained bulky fibers with a very compact structure. The results showed that after treatment with PPC domains, bulky collagen fibers ($>10 \mu$ m) broke off and became microfibrils with diameters of 2 to 5 μ m or fibrils with diameters of approximately 2 μ m (Fig. 7). When observed at high magnifications ($\times 150,000$ to $\times 200,000$), the collagen treated with PPC domains was dissociated into thinner fibrils or microfibrils (Fig. 7 and S6). The diameters of the collagen monomers were approximately 1.5 nm, and the microfibril diameters were approximately 16 to 30 nm (Fig. 7C). PPC-treated collagen was arranged loosely in a network-like structure. After incubation with 6 M urea at 37°C for 12 h, the surface of type I collagen became irregular and appeared to melt but remained compact (see Fig. S7), which suggested that urea swelled collagen through a different mechanism than PPC domains. Collagen monomers could not be observed, because collagen monomers are soluble and were removed during sample preparation. The results indicated that PPC domains were able to swell the aggregate structures of collagen. Notably, there were many vesicular projections, with diameters of approximately 15 to 30 nm, on the surface of PPC-treated collagen, suggesting that collagen fibrils might begin to inflate at these sites (Fig. 7 and S6, red arrows).

Pyridinoline, also known as hydroxylsypylpyridinoline, is a fluorescent cross-linking compound for collagen fibers (34, 35). When collagen is dissolved in solution, the

FIG 8 Fluorescence emission spectra of collagen in different reaction systems. (A) Insoluble type I collagen (5 mg) was incubated in 1 ml PBS (20 mM, pH 7.4) at 37°C for 1 h and 5 h and with 6 M urea for 5 h with gentle agitation. (B) Collagen was incubated in 1 ml PBS with 0.15 μ M GST at 37°C for 1 h and 5 h, with slight gentle agitation. (C to L) Collagen was added to 1 ml PBS with 0.15 μ M recombinant GST-PPC fusion proteins and then incubated at 37°C for 1 h and 5 h with gentle agitation. The samples were excited at 295 nm, and the emission was monitored at between 350 and 500 nm.

fluorescence of pyridinoline cross-linking collagen can be measured at 410 nm following excitation at 295 nm using a spectrofluorometer. The fluorescence emission spectra of insoluble type I collagen are shown in Fig. 8. After incubating collagen with phosphate-buffered saline (PBS; 20 mM, pH 7.4) (Fig. 8A) and GST protein (Fig. 8B) at 37°C for 1 or 5 h, there were minimal emission peaks at 410 nm. After incubation with PPC domains at 37°C for 1 h, the emission peak was initially low; however, after incubation for 5 h, the PPC-treated emission peaks increased with increased soluble collagen (Fig. 8B to L). This result suggested that PPC domains promote the dissolution of insoluble type I collagen and did not disrupt the pyridinoline cross-links. However, after incubation with 6 M urea for 1 h, the peak completely disappeared (Fig. 8A). Combined with the SEM results (Fig. S7), these results suggest that urea and PPC domains swell collagen through different mechanisms.

Trypsin has gelatinase activity and is therefore able to hydrolyze denatured collagen. In our study, trypsin could not hydrolyze normal collagen (Fig. 9, bar 1) or PPC-swelled

FIG 9 Activities of trypsin against untreated collagen (1), E423-PPC12-treated collagen (2), and thermally denatured collagen (3). Thermally denatured collagen was prepared by heating insoluble type I collagen at 65°C for 20 min. PPC-treated collagen was prepared by incubating insoluble type I collagen with 0.15 μ M PPC at 37°C for 5 h. Trypsin activity was measured at 37°C for 3 h using the ninhydrin-based method. Values are expressed as the means $\pm$ SDs ($n = 3$).

collagen (Fig. 9, bar 2), whereas thermally denatured collagen was degraded by trypsin (Fig. 9, bar 3). This result suggested that the triple-helix structure of collagen was not unwound by PPC domains. The SEM results also showed that PPC domains exposed collagen fibrils but did not unwind the triple-helix structure.

Determination of the key residues in the PPC domain of EmpA. (i) Sequence alignment and interaction surface prediction. The sequences of the eight PPC domains used in this study were aligned to analyze the conserved amino acid residues. Phylogenetic analysis and sequence alignment (Fig. 10A and B) showed that the sequences of PPC domains from different bacteria had low identities but contained several consensus sites. These sites might play important roles in the maintenance of PPC domain structure and function. Because EmpA-PPC had the strongest collagen-swelling and substrate-binding abilities, we chose to use EmpA-PPC during our PPC domain mutation experiment. Sequence alignment showed that the polar and aromatic residues Y6, D26, D28, Y30, W42, E53, C55, and Y65 were conserved amino acids. Many studies have confirmed that polar and aromatic amino acids play key roles in protein binding. These amino acid residues easily form ionic or hydrogen bonds. Furthermore, the interaction surface prediction (Fig. 10D and E) showed that there were 27 amino acid residues on the interaction surface of EmpA-PPC. Residues Y6, V10, S12, V18, Y30, E53, C55, I57, and Y65 appeared on the interaction surface. Among these, Y6, V10, V18, Y30, I57, and Y65 were relatively well-conserved amino acid residues ($>50\%$), and the aromatic residues Y6, Y30, and Y65 and the hydrophobic residues V10, V18, and I57 interact directly with collagen (Fig. 10E), suggesting that these amino acid residues might play important roles in collagen binding. In addition, sequence alignment also found that these three hydrophobic sites (V10, V18, and I57) also contained hydrophobic amino acids in different PPC domains. Hydrophobic force might be the source of binding strength in the PPC-collagen interaction. To confirm the key amino acid residues of the PPC domain and to explore the interaction between the PPC domain and collagen, the Y6A, V10G, S12I, V18G, D26N, D28N, Y30A, W42A, E53Q, C55A, I57A, Y65A, and S12I/D26N mutants were expressed as GST fusion proteins (see Fig. S8).

(ii) Analysis of the binding abilities of PPC domain mutants. As shown in Fig. 11, the D26N, D28N, W42A, E53Q, and S12I/D26N mutants lost their abilities to bind collagen, feather meal, and chitin. The mutants with mutations on the interaction surface, Y6A, V10G, V18G, Y30A, I57A, and Y65A, had weak abilities to bind collagen, feather meal, and chitin. The V10G, V18G, Y30A, and I57A mutants completely lost their collagen-binding abilities. Interestingly, the collagen-binding ability of the C55A mutant was 2-fold that of EmpA-PPC, and the chitin-binding ability was improved by approximately 3-fold that of EmpA-PPC, for unknown reasons. These results suggested

FIG 11 Effects of site-directed mutagenesis on the substrate binding abilities of PPC domains. The bar graph represents the relative values of the recombinant GST-PPC fusion proteins. Collagen, feather meal, and chitin were used as the substrates. Values are expressed as the means $\pm$ SDs ($n = 3$).

discovered that human epidermal growth factor containing CBD (EGF-CBD fusion protein) could be used as a healing agent for wound tissue (36). Similarly to CBD, the PPC domain might also be used in targeted therapy. The EmpA-PPC C55A mutant, with better collagen-binding ability, might be more suitable for applications in medical industries than the wild-type PPC domain.

(iii) Collagen-swelling abilities of the mutants. Viewed macroscopically, the Y6A, V10G, S12I, V18G, D26N, D28N, Y30A, W42A, E53Q, C55A, I57A, and Y65A mutants retained the ability to swell collagen but had weaker collagen-swelling abilities than wild-type EmpA-PPC. Among them, V10G, V18G, C55A, and Y65A showed great reductions in their collagen-swelling abilities. S12I/D26N was nearly unable to swell collagen (Fig. 12). Y6, V10, V18, Y30, I57, and Y65 are located at the interaction surface of the EmpA-PPC domain. Mutations in these amino acid sites might disrupt the binding between the PPC domain and collagen fibers. In addition, tyrosine (Y) and tryptophan (W), which both contain large side chain groups, might help maintain direct interactions by forming hydrogen bonds and hydrophobic bonds with the interaction partner (37). Aspartate (D) and glutamic acid (E) might interact with peptide ligands by forming direct salt bridges (38). Cysteine (C) might maintain the structure of the protein through hydrogen bonds or disulfide bonds (37).

Molecular dynamic simulation of the PPC domain. The homologous modeling results showed that PPC domains were immunoglobulin-like (Ig-like) beta-sandwich domains (Fig. 13B). Although the amino acid sequences of the PPC domains were quite different, their spatial structures were very similar. To identify the protein interaction patterns between different PPC domains and collagen, ligand docking simulations were performed using Accelrys Discovery Studio version 2.6 (39). As shown in Fig. 13, most PPC domains attached to the sides of collagen monomers, except for VVP-PPC and EmpA-PPC, which bound to one end of the collagen monomer. The hydrophobic binding pockets of the PPC domains were partially overlaid and were identified as the binding sites in the docking study (Fig. 13). PPC domains might have similar binding modes for collagen fibers, and the hydrophobic area of PPC domains might be important components of collagen binding.

FIG 12 The collagen-swelling effect of PPC mutants. (Top) Insoluble type I collagen (5 mg) in 2 ml 20 mM PBS buffer (pH 7.4) with 0.15 μ M GST-EmpA-PPC or 0.15 μ M site-directed mutants were incubated for 12 h at 37°C. Collagen incubated with PBS was used as a control. (Bottom) Statistical analysis of the relative collagen volumes of untreated and treated collagen fascicles. The bands were quantitated using ImageJ 1.46. Values are expressed as the means $\pm$ SDs, taking the average from three measurements.

SEM, spectroscopy analysis, and thermal unfolding experiments showed that PPC domains were able to swell the aggregate structures of collagen and expose collagen monomers but did not disrupt the pyridinoline cross-links between collagen monomers or unwind the collagen triple helix. A molecular dynamic study showed that PPC domains might bind to collagen in two ways and that collagen binds to the hydrophobic areas of PPC domains (Fig. 13).

Conclusions. PPC domains exist in the C termini of a wide variety of proteases secreted by marine bacteria. This study systematically explored the substrate-binding and collagen-swelling abilities of PPC domains and revealed the functional mechanism underlying PPC domain-mediated collagen swelling. PPC domains bind not only protein substrates (collagen and keratin) but also polysaccharides (chitin and cellulose). The PPC domain could clearly swell insoluble type I collagen. Site-directed mutagenesis showed that the conserved polar and aromatic residues in PPC domains might play key roles in the binding and swelling of collagen. Collagen fibers have been widely used to manufacture biodegradable scaffolds and hemostatic sponges; however, the low porosity of stents and collagen sponges has limited the distribution of the extracellular matrix and the growth of homogenous cells (40, 41). The collagen-binding and swelling capacities of PPC can be used to modify collagen fibers and create novel stents or sponges with high porosity ratios and good physical properties, which might be useful for facilitating cell proliferation and promoting tissue repair. Therefore, the collagen-

FIG 13 (A) Docking simulations for the eight PPC domains with the collagen molecule. (B) Homology modeling and surface charge predictions of the PPC domains. Each residue is colored according to its hydrophobicity, as indicated by the color code bar, with the following relative hydrophobicity values: red (negative number) represents high hydrophilicity and blue (positive number) represents high hydrophobicity. Electrostatic surfaces are shown in red (negative number) for negative charges, blue (positive number) for positive charges, and white for neutral charges.

swelling effects of PPC domains may demonstrate great potential for future use in medicine, pharmacy, cosmetics, and the food industry. These results increase our understanding of the functions and biological significance of PPC domains and help us to explore novel biotechnologies and applications for the PPC domain.

MATERIALS AND METHODS

Materials. All PPC domains used in this report are listed in Table 1. The *Pseudoalteromonas* sp. CSN423, *Pseudoalteromonas* sp. J2, *Vibrio anguillarum*, and *V. vulnificus* strains used in this study were originally isolated from the in-shore environment of the South China Sea (18°29'1980"N, 109°34'761"E) (42). The strain *Salinivibrio* sp. YH4 was originally isolated from the Yuncheng Salt Lake in Shanxi Province, China. Bacterial extracellular proteases containing PPC domains in those strains were detected in this study. The sequences of those genes were submitted to GenBank (Table 1). The sequences of protease from *V. anguillarum* and *V. vulnificus* strains are the same as metalloprotease EmpA from *V. anguillarum* (Q6T863) and metalloprotease VVP from *V. vulnificus* (Q06AK2), respectively (17, 18). *Escherichia coli* DH5 α and *E. coli* BL21(DE3) were purchased from Novagen (USA) and cultured at 37°C in LB liquid medium supplemented with ampicillin for the selection of transformants. Restriction endonuclease, *Pfu* DNA polymerase, ampicillin, isopropyl- β -D-thiogalactopyranoside (IPTG), and ligase mix were purchased from Thermo Fisher Oxoid (Basingstoke, Hampshire, UK). Collagenase, crude type I, ≥ 125 CDU/mg, from *Clostridium histolyticum* (C0130) was purchased from Sigma. Soluble and insoluble type I collagen (bovine tendon collagen, number M3809) was purchased from Worthington Biochemical Co. (USA). ANS was purchased from Sigma-Aldrich and used without further purification. All other reagents were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China).

Alignments and phylogenetic analysis. The protein sequences of the PPC domains were searched and retrieved from the NCBI database based on the sequences of the PPC domains from *Pseudoalteromonas* sp. CSN423, *Pseudoalteromonas* sp. J2, *V. anguillarum*, *V. vulnificus*, and *Salinivibrio* sp. YH4. Nonredundant protein sequences (nr) and the BLASTp (protein-protein BLAST) algorithm were used as the search settings. On the basis of our BLAST searches, a total of 440 PPC domains in 34 bacterial species were identified. These data sets provided a solid basis for a comprehensive analysis of the phylogenetic relationships of the PPC domains. All PPC amino acid sequences were aligned using ClustalX as implemented in MEGA 6.0 software. The evolutionary distances of PPC domains were generated with MEGA 6.0. The network was drawn using Gephi software, which was used to analyze and visualize sequence comparisons.

Expression and purification of the GST-fused PPC domains. Glutathione *S*-transferase (GST) and the fusion GST-PPC proteins were expressed and purified using previously described methods (3). The PPC domains were amplified using PCR, with bacterial genomic DNA as the template. The PCR products were ligated with EcoRI/XhoI-linearized pGEX-4T-1 vectors (Pharmacia Biotech Inc., USA) to construct

TABLE 2 Oligonucleotide primers used to amplify PPC domains and their mutants

Primer	Sequence (5'→3') ^a
VVP-PPC-Eco	CGGAATTCTCGGAAGTATTCTATACCTTTAC
VVP-PPC-Xho	CCGCTCGAGATATTGAAGCTTTAACGTCACAC
YHM-PPC-Eco	CGGAATTCTCTTCTGAGCTATTCACT
YHM-PPC-Xho	CCGCTCGAGGTTGCGAACCAAGCTTACAC
YHS-PPC-Eco	CGGAATTCTCGAGCGCAACGCTTTTCTAC
YHS-PPC-Xho	CCGCTCGAGATATCGTGCTGTGACGCTTAC
E423-PPC1-Eco	CGGAATTCGAGCAGTTGTTTTTAC
E423-PPC1-Xho	CCGCTCGAGTGCCTCTACCATAACG
E423-PPC2-Eco	CGGAATTCGGTTGGACACGCTTTAC
E423-PPC2-Xho	CCGCTCGAGACCTTGTAAGTCTATATACC
J2-PPC1-Eco	CGGAATTCGAGCAACTATTCTTTACTTTGG
J2-PPC1-Xho	CCGCTCGAGTGCTTCAACCATTACATAGTAAG
J2-PPC2-Eco	CGGAATTCCTTACTCAAGATTATCAGAAGG
J2-PPC2-Xho	CCGCTCGAGACCTCTTAAATCAATGTACCAAG
EmpA-PPC-Eco	CGGAATTCAGCTCCGAAGCCTTCTATACCT
EmpA-PPC-Xho	CCGCTCGAGATCCAGTCTTAACGTTACACC
emp-Y6A-Eco	CGGAATTCAGCTCCGAAGCCTTCGCTACCTTTACG
emp-D26N	TTAGTTTGGGCTCAGGTAACGCTGATTGTATGTC
emp-D26N-ANTI	ACATACAAATCAGCGTTACCTGAGCCCAAACCTAATC
emp-D28N	AGTTTGGGCTCAGGTGATGCTAACTTGATGTCAAAGCCG
emp-D28N-ANTI	CGGCTTTGACATACAAGTTAGCATCACCTGAGCCCAAACCT
emp-Y30A	AGGTGATGCTGATTGGCTGTCAAAGCCGGCAGC
emp-Y30A-ANTI	GCTGCCGGCTTTGACAGCCAAATCAGCATCACCT
emp-W42A	AACCAACCCTTCTTCGGCTGATTGTCGCCCTTAC
emp-W42A-ANTI	GTAAGGCGGACAAATCAGCCGAAGAAGTGTTGGT
emp-E53Q	CTTACAAATCGGGCAACAATCAGCAGTGCACAATTTCTGC
emp-E53Q-ANTI	GCAGAAATTGTGACTGCTGATTGTTGCCGATTTGTAAG
emp-C55A	ATCGGGCAACAATGAACAGGCTACAATTTCTGCAACACCAG
emp-C55A-ANTI	CTGGTGTTCAGAAATTGTAGCCTGTTTATTGTTGCCCGAT
emp-Y65A	CTGCAACACCAGGAACCAACCGCTCATGTGATGCTCAAAGGC
emp-Y65A-ANTI	GCCTTTGAGCATCACATGAGCGGTGGTTCCTGGTGTTCAG

^aEcoRI/XhoI restriction sites in the primers are underlined.

plasmids for the expression of GST-fused PPC domains (Table 1; see also Fig. S4 in the supplemental material). All of the expression plasmids were transformed into *E. coli* BL21(DE3) competent cells. Expression was induced with 0.2 mM IPTG at 15°C for 16 h. The GST-fused PPC domains were purified by glutathione agarose chromatography (GE Healthcare, USA).

Site-directed mutagenesis of the PPC domains. Site-directed mutagenesis was performed by overlapping extension PCR using the expression plasmids constructed as described above (pGEX-EmpA-PPC) as the templates. EmpA-PPC mutants (Y6A, V10G, S12I, V18G, D26N, D28N, Y30A, W42A, E53Q, C55A, I57A, Y65A, and S12I/D26N) were introduced by primers containing single mutations (Table 2). The mutated genes were subcloned into pGEX-4T-1 and transformed into *E. coli* BL21(DE3). After confirmation by enzyme digestion and nucleotide sequencing, all mutants were expressed and purified using the same condition as for GST-fused PPC domains.

Insoluble substrate binding assay. Insoluble substrates (insoluble type I collagen, feather meal, and chitin, 1 mg) were added to 100 μ l PBS (20 mM, pH 7.4) containing recombinant proteins and incubated at 37°C for 2 h with stirring. Recombinant protein in PBS without any other substrate was used as the control. After incubation, the mixtures were centrifuged at 13,000 $\times g$ for 3 min. The supernatants were removed for 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Coomassie-stained gels were scanned using an Epson Perfection V500 scanner (Seiko Epson, Japan), and the proteins in the gels were quantitated using ImageJ 1.46 software (NIH, USA).

Binding ability of the soluble substrate by ANS detection assay. The binding affinities of the PPC domains were determined using ANS fluorescence detection (25). The fluorescence emission experiments were performed for the PPC domains in the presence of a 40 μ M 8-anilino-1-naphthalenesulfonic acid (ANS) probe. The PPC domain was exposed to soluble collagen and carboxymethylcellulose, which were dissolved at a concentration of 1 mg/ml with 20 mM PBS (pH 7.4). Two hundred fifty microliters PPC domain solution (0.15 μ M) and 250 μ l biomacromolecules (1.0 mg/ml) were mixed and incubated at 25°C for 10 min in a final volume of 0.5 ml. Then, 0.25 μ l ANS (8.0 mM) was added to the mixed solutions at 25°C for 30 s with stirring. The fluorescence spectra were measured from the samples containing ANS, with excitation at 374 nm and emission at 485 nm, in an EnSpire 2300 microplate reader (Perkin Elmer, Waltham, MA, USA). The background from the buffer solution was subtracted from all fluorescence emission measurements.

Electrophoretic mobility shift assay. The 16S rRNA gene was amplified using a universal primer set (27F, 5'-AGAGTTTGATCCTGGCTCAG-3'; and 1492R, 5'-ACGGCTACCTTGTTACGACTT-3'), with the genomic DNA of *V. anguillarum* as the template. The EMSA was performed in a 20- μ l reaction mixture containing 25 ng/ μ l 16S rRNA and 0.075 μ M GST-PPC. A total of 10 μ l 16S rRNA (50 ng/ μ l) was incubated with 10 μ l

0.15 μ M GST-PPC recombinant proteins at room temperature for 20 min and then electrophoresed for 30 min at a constant voltage of 120 V on 1.0% agarose gel. As a control, 20 μ l 25 ng/ μ l 16S rRNA and 20 μ l 0.075 μ M GST-PPC were electrophoresed.

Swelling of insoluble type I collagen by PPC domain. A total of 5 mg of insoluble type I collagen in 2 ml PBS (20 mM, pH 7.4) was mixed with 0.15 μ M GST, 0.15 μ M GST-PPC domains, 0.15 μ M GST-EmpA-PPC mutants, or 6 M urea, in tubes, to investigate the effects of PPC domains on collagen swelling. The samples were incubated at 37°C for 12 h with continuous stirring and then photographed using a digital camera (Canon, USA). Then, based on the area of the collagen in the pictures, collagen swelling was determined with ImageJ 1.46 software (NIH, USA). Values are expressed as the means $\pm$ standard deviations (SDs).

Pyridinoline cross-linking fluorescence spectrophotometric measurements. Insoluble type I collagen (5 mg) was added to 1 ml PBS (20 mM, pH 7.4) containing 0.15 μ M recombinant fusion proteins or 6 M urea and then incubated at 37°C for 1 h or 5 h with continuous stirring. Then, the supernatant was collected and fluorescence spectra were detected by scanning on a F2700 spectrofluorometer (Hitachi, Japan) at room temperature. The samples were excited at 295 nm, and the emission was monitored at between 350 and 500 nm, with bandwidths of 5 nm for excitation and 10 nm for emission.

Scanning electron microscopy. A total of 5 mg type I insoluble collagen was incubated with 0.15 μ M GST, 0.15 μ M GST-PPC domains, 0.15 μ M GST-EmpA-PPC mutants, or 6 M urea in 2 ml PBS (20 mM, pH 8.5) at 37°C for 12 h with continuous stirring. After rinsing three times with sterile water, the vacuum freeze-drying method was used to dehydrate water from treated collagen samples. The samples were observed using a scanning electron microscope (Helios NanoLab 600i).

Enzyme assays. The activities of trypsin against insoluble type I collagen, thermally denatured collagen, and PPC-pretreated collagen were measured at 37°C for 3 h with the ninhydrin-based method. Thermally denatured collagen was prepared by heating insoluble type I collagen at 65°C for 20 min. PPC-pretreated collagen was prepared by incubating insoluble type I collagen with 0.15 μ M GST-PPC at 37°C for 5 h. The collagenase activities for insoluble type I collagen or PPC-pretreated collagen samples were analyzed by the method described by Lowry et al. (43).

Homology searches and molecular docking simulations. The secondary and tertiary structures of the PPC domain were modeled using the secondary structure prediction server Jpred (<http://www.compbio.dundee.ac.uk/jpred/>) (44) and the three-dimensional (3D) structure prediction server Phyre2 (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>) (45). The confidences of the PPC domain structure predictions all were larger than 90%. The 3D structures of the PPC domains were built with homology modeling, using the structure of the metalloprotease vEP C-ter 100 from *V. vulnificus* as the template (PDB code 2LUW) (16). The crystal structure of a collagen peptide (PDB code 1CGD) has been determined and showed a collagen triple helix defined by the supercoiling of three polypeptide chains (46). The binding sites between the PPC domains and collagen were identified using Accelrys Discovery Studio version 2.6 (39). The structures of the PPC domains were used in simulations and treated as a static macromolecule, whereas collagen was treated as flexible molecules. Simulations were conducted with binding grids covering the whole protein surface to include all possible binding sites. The collagen binding sites were selected based on the lowest binding energy.

Statistical analysis. Statistical evaluation was performed by using Origin 9.1 software. To confirm a statistically significant difference between the mean values of the two groups, the Student's *t* test was applied. A *P* value of <0.05 was considered to indicate a significant difference between two groups. For the derivate values, the combined standard error formula was used to calculate the standard deviations. All values were plotted as the mean values $\pm$ standard deviations.

Accession number(s). The sequences corresponding to bacterial extracellular proteases containing PPC domains in *Pseudoalteromonas* sp. J2 and *Salinivibrio* sp. YH4 were submitted to GenBank and are shown in Table 1.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00611-19>.

SUPPLEMENTAL FILE 1, PDF file, 1.7 MB.

ACKNOWLEDGMENTS

The work was supported by the National Natural Science Foundation of China (31370104, 21205142, and 31400002), the Hunan Provincial Natural Science Foundation of China (2018JJ2497), the Opening Foundation of the Chinese National Engineering Research Center for Control and Treatment of Heavy Metal Pollution (number 2015CNERC-CTHMP-07), the Hunan Provincial Innovation Foundation For Postgraduates (CX2017B074), and the Fundamental Research Funds for the Central Universities of Central South University (2018zzts119, 2018zzts392, 2017zzts076, and 2017zzts351).

We declare no conflicts of interest.

REFERENCES

- Chen XL, Xie BB, Bian F, Zhao GY, Zhao HL, He HL, Zhou BC, Zhang YZ. 2009. Ecological function of myroilysin, a novel bacterial M12 metalloprotease with elastinolytic activity and a synergistic role in collagen hydrolysis, in biodegradation of deep-sea high-molecular-weight organic nitrogen. *Appl Environ Microbiol* 75:1838–1844. <https://doi.org/10.1128/AEM.02285-08>.
- Zhan Y, Chen F. 16 December 2018. Bacteriophages that infect marine roseobacters: genomics and ecology. *Environ Microbiol* <https://doi.org/10.1111/1462-2920.14504>.
- He HL, Guo J, Chen XL, Xie BB, Zhang XY, Yu Y, Chen B, Zhou BC, Zhang YZ. 2012. Structural and functional characterization of mature forms of metalloprotease E495 from Arctic sea-ice bacterium *Pseudoalteromonas* sp. SM495. *PLoS One* 7:e35442. <https://doi.org/10.1371/journal.pone.0035442>.
- Zhou MY, Wang GL, Li D, Zhao DL, Qin QL, Chen XL, Chen B, Zhou BC, Zhang XY, Zhang YZ. 2013. Diversity of both the cultivable protease-producing bacteria and bacterial extracellular proteases in the coastal sediments of King George Island, Antarctica. *PLoS One* 8:e79668. <https://doi.org/10.1371/journal.pone.0079668>.
- Ran L-Y, Su H-N, Zhou M-Y, Wang L, Chen X-L, Xie B-B, Song X-Y, Shi M, Qin Q-L, Pang X, Zhou B-C, Zhang Y-Z, Zhang X-Y. 2014. Characterization of a novel subtilisin-like protease myroicolsin from deep sea bacterium *Myroides profundus* D25 and molecular insight into its collagenolytic mechanism. *J Biol Chem* 289:6041–6053. <https://doi.org/10.1074/jbc.M113.513861>.
- Ran LY, Su HN, Zhao GY, Gao X, Zhou MY, Wang P, Zhao HL, Xie BB, Zhang XY, Chen XL, Zhou BC, Zhang YZ. 2013. Structural and mechanistic insights into collagen degradation by a bacterial collagenolytic serine protease in the subtilisin family. *Mol Microbiol* 90:997–1010. <https://doi.org/10.1111/mmi.12412>.
- Hasan NA, Grim CJ, Lipp EK, Rivera ING, Chun J, Haley BJ, Taviani E, Choi SY, Hoq M, Munk AC, Brettin TS, Bruce D, Challacombe JF, Detter JC, Han CS, Eisen JA, Huq A, Colwell RR. 2015. Deep-sea hydrothermal vent bacteria related to human pathogenic *Vibrio* species. *Proc Natl Acad Sci U S A* 112:E2813–E2819. <https://doi.org/10.1073/pnas.1503928112>.
- Huang J, Zeng B, Liu D, Wu R, Zhang J, Liao B, He H, Bian F. 2018. Classification and structural insight into vibriolysin-like proteases of *Vibrio* pathogenicity. *Microb Pathog* 117:335–340. <https://doi.org/10.1016/j.micpath.2018.03.002>.
- Lee SJ, Jung YH, Ryu JM, Jang KK, Choi SH, Han HJ. 2016. VvpE mediates the intestinal colonization of *Vibrio vulnificus* by the disruption of tight junctions. *Int J Med Microbiol* 306:10–19. <https://doi.org/10.1016/j.ijmm.2015.10.006>.
- Wang YK, Zhao GY, Li Y, Chen XL, Xie BB, Su HN, Lv YH, He HL, Liu H, Hu J, Zhou BC, Zhang YZ. 2010. Mechanistic insight into the function of the C-terminal PKD domain of the collagenolytic serine protease desesin MCP-01 from deep sea *Pseudoalteromonas* sp. SM9913: binding of the PKD domain to collagen results in collagen swelling but does not unwind the collagen triple helix. *J Biol Chem* 285:14285–14291. <https://doi.org/10.1074/jbc.M109.087023>.
- Xu ZS, Du X, Li TT, Gan F, Tang B, Tang XF. 2011. Functional insight into the C-terminal extension of halolysin SptA from haloarchaeon *Natrinema* sp. J7. *PLoS One* 6:e23562. <https://doi.org/10.1371/journal.pone.0023562>.
- Huang J, Wu C, Liu D, Yang X, Wu R, Zhang J, Ma C, He H. 2017. C-terminal domains of bacterial proteases: structure, function and the biotechnological applications. *J Appl Microbiol* 122:12–22. <https://doi.org/10.1111/jam.13317>.
- Bauer R, Wilson JJ, Philominathan ST, Davis D, Matsushita O, Sakon J. 2013. Structural comparison of ColH and ColG collagen-binding domains from *Clostridium histolyticum*. *J Bacteriol* 195:318–327. <https://doi.org/10.1128/JB.00010-12>.
- Katikaneni R, Ponnappakkam T, Matsushita O, Sakon J, Gensure R. 2014. Treatment and prevention of chemotherapy-induced alopecia with PTH-CBD, a collagen-targeted parathyroid hormone analog, in a non-depilated mouse model. *Anticancer Drugs* 25:30–38. <https://doi.org/10.1097/CAD.0b013e3283650bfb>.
- Zhang YZ, Ran LY, Li CY, Chen XL. 2015. Diversity, structures, and collagen-degrading mechanisms of bacterial collagenolytic proteases. *Appl Environ Microbiol* 81:6098–6107. <https://doi.org/10.1128/AEM.00883-15>.
- Yun JH, Kim H, Park JE, Lee JS, Lee W. 2013. Solution structure and dynamics of C-terminal regulatory domain of *Vibrio vulnificus* extracellular metalloprotease. *Biochem Biophys Res Commun* 430:541–546. <https://doi.org/10.1016/j.bbrc.2012.11.117>.
- Miyoshi S. 2013. Extracellular proteolytic enzymes produced by human pathogenic *Vibrio* species. *Front Microbiol* 4:339. <https://doi.org/10.3389/fmicb.2013.00339>.
- Elgaml A, Miyoshi SI. 2017. Regulation systems of protease and hemolysin production in *Vibrio vulnificus*. *Microbiol Immunol* 61:1–11. <https://doi.org/10.1111/1348-0421.12465>.
- Edwin A, Rompikuntal P, Bjorn E, Stier G, Wai SN, Sauer-Eriksson AE. 2013. Calcium binding by the PKD1 domain regulates interdomain flexibility in *Vibrio cholerae* metalloprotease PrtV. *FEBS Open Bio* 3:263–270. <https://doi.org/10.1016/j.fob.2013.06.003>.
- Sorimachi H, Hata S, Ono Y. 2011. Calpain chronicle—an enzyme family under multidisciplinary characterization. *Proc Jpn Acad Ser B Phys Biol Sci* 87:287–327. <https://doi.org/10.2183/pjab.87.287>.
- Nagae M, Hirata T, Morita-Matsumoto K, Theiler R, Fujita M, Kinoshita T, Yamaguchi Y. 2016. 3D structure and interaction of p24beta and p24delta Golgi dynamics domains: implication for p24 complex formation and cargo transport. *J Mol Biol* 428:4087–4099. <https://doi.org/10.1016/j.jmb.2016.08.023>.
- Xu K, Tzvetkova-Robev D, Xu Y, Goldgur Y, Chan YP, Himanen JP, Nikolov DB. 2013. Insights into Eph receptor tyrosine kinase activation from crystal structures of the EphA4 ectodomain and its complex with ephrin-A5. *Proc Natl Acad Sci U S A* 110:14634–14639. <https://doi.org/10.1073/pnas.1311000110>.
- Cristobal HA, Lopez MA, Kothe E, Abate CM. 2011. Diversity of protease-producing marine bacteria from sub-antarctic environments. *J Basic Microbiol* 51:590–600. <https://doi.org/10.1002/jobm.201000413>.
- Nitahara S, Kato S, Usui A, Urabe T, Suzuki K, Yamagishi A. 2017. Archaeal and bacterial communities in deep-sea hydrogenetic ferromanganese crusts on old seamounts of the northwestern Pacific. *PLoS One* 12:e0173071. <https://doi.org/10.1371/journal.pone.0173071>.
- Huang JF, Wu RB, Wu CL, Liu D, Zhang J, Liao BQ, Lei M, Xiao X, Ma CB, He HL. 2018. New method of detecting hydrophobic interaction between C-terminal binding domain and biomacromolecules. *J Biotechnol* 265:101–108. <https://doi.org/10.1016/j.jbiotec.2017.11.009>.
- Herzog B, Overly DP, Haltli B, Kerr RG. 2016. Discovery of keratinases using bacteria isolated from marine environments. *Syst Appl Microbiol* 39:49–57. <https://doi.org/10.1016/j.syapm.2015.10.004>.
- Blank CE, Parks RW, Hinman NW. 2016. Chitin: a potential new alternative nitrogen source for the tertiary, algal-based treatment of pulp and paper mill wastewater. *J Appl Phycol* 28:2753–2766. <https://doi.org/10.1007/s10811-016-0808-5>.
- Pavlovic A, Krausko M, Adamec L. 2016. A carnivorous sundew plant prefers protein over chitin as a source of nitrogen from its traps. *Plant Physiol Biochem* 104:11–16. <https://doi.org/10.1016/j.plaphy.2016.03.008>.
- Usha R, Ramasami T. 2002. Effect of hydrogen-bond-breaking reagent (urea) on the dimensional stability of rat tail tendon (RTT) collagen fiber. *J Appl Polym Sci* 84:975–982. <https://doi.org/10.1002/app.10262>.
- Grant GA, Henderson KO, Eisen AZ, Bradshaw RA. 1980. Amino acid sequence of a collagenolytic protease from the hepatopancreas of the fiddler crab, *Uca pugnator*. *Biochemistry* 19:4653–4659. <https://doi.org/10.1021/bi00561a018>.
- Sarkar SK, Marmer B, Goldberg G, Neuman KC. 2012. Single-molecule tracking of collagenase on native type I collagen fibrils reveals degradation mechanism. *Curr Biol* 22:1047–1056. <https://doi.org/10.1016/j.cub.2012.04.012>.
- Caviness P, Bauer R, Tanaka K, Janowska K, Roeser JR, Harter D, Sanders J, Ruth C, Matsushita O, Sakon J. 2018. Ca²⁺-induced orientation of tandem collagen binding domains from clostridial collagenase ColG permits two opposing functions of collagen fibril formation and retardation. *FEBS J* 285:3254–3269. <https://doi.org/10.1111/febs.14611>.
- Sekiguchi H, Uchida K, Matsushita O, Inoue G, Nishi N, Masuda R, Hamamoto N, Koide T, Shoji S, Takaso M. 2018. Basic fibroblast growth factor fused with tandem collagen-binding domains from *Clostridium histolyticum* collagenase ColG increases bone formation. *BioMed Res Int* 2018:8393194. <https://doi.org/10.1155/2018/8393194>.
- Kinnunen J, Kokkonen HT, Kovanen V, Hauta-Kasari M, Vahimaa P,

- Lammi MJ, Töyräs J, Jurvelin JS. 2012. Nondestructive fluorescence-based quantification of threose-induced collagen cross-linking in bovine articular cartilage. *J Biomed Opt* 17:97003. <https://doi.org/10.1117/1.JBO.17.9.097003>.
35. Yan H, Chen Y, Zhang J, Liu W, Chen R. 2016. The role of free radicals in the photodynamic treatment of fibrotic skin diseases. *Adv Exp Med Biol* 923:69–74. https://doi.org/10.1007/978-3-319-38810-6_10.
 36. Kim DG, Kim EY, Kim YR, Kong IS. 2015. Construction of chimeric human epidermal growth factor containing short collagen-binding domain moieties for use as a wound tissue healing agent. *J Microbiol Biotechnol* 25:119–126. <https://doi.org/10.4014/jmb.1405.05073>.
 37. Weigt M, White RA, Szurmant H, Hoch JA, Hwa T. 2009. Identification of direct residue contacts in protein-protein interaction by message passing. *Proc Natl Acad Sci U S A* 106:67–72. <https://doi.org/10.1073/pnas.0805923106>.
 38. Lotze S, Bakker HJ. 2015. Structure and dynamics of a salt-bridge model system in water and DMSO. *J Chem Phys* 142:212436. <https://doi.org/10.1063/1.4918904>.
 39. Schotland P, Bojunga N, Zien A, Trame MN, Lesko LJ. 2016. Improving drug safety with a systems pharmacology approach. *Eur J Pharm Sci* 94:84–92. <https://doi.org/10.1016/j.ejps.2016.06.009>.
 40. Ng KW, Khor HL, Huttmacher DW. 2004. *In vitro* characterization of natural and synthetic dermal matrices cultured with human dermal fibroblasts. *Biomaterials* 25:2807–2818. <https://doi.org/10.1016/j.biomaterials.2003.09.058>.
 41. Santos TDS, Abuna RPF, Almeida ALGD, Beloti MM, Rosa AL. 2015. Effect of collagen sponge and fibrin glue on bone repair. *J Appl Oral Sci* 23:623–628. <https://doi.org/10.1590/1678-775720150374>.
 42. Wu C, Liu D, Yang X, Wu R, Zhang J, Huang J, He H. 2016. Improving production of protease from *Pseudoalteromonas* sp. CSN423 by random mutagenesis. *Mar Biotechnol* 18:610–618. <https://doi.org/10.1007/s10126-016-9721-9>.
 43. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275.
 44. Drozdetskiy A, Cole C, Procter J, Barton GJ. 2015. JPred4: a protein secondary structure prediction server. *Nucleic Acids Res* 43:W389–W394. <https://doi.org/10.1093/nar/gkv332>.
 45. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJ. 2015. The Phyre2 web portal for protein modeling, prediction and analysis. *Nat Protoc* 10:845–858. <https://doi.org/10.1038/nprot.2015.053>.
 46. Bella J, Brodsky B, Berman HM. 1995. Hydration structure of a collagen peptide. *Structure* 3:893–906. [https://doi.org/10.1016/S0969-2126\(01\)00224-6](https://doi.org/10.1016/S0969-2126(01)00224-6).