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ORIGINAL PAPER

Molecular analysis of the gene encoding a cold-adapted halophilic subtilase from deep-sea psychrotolerant bacterium *Pseudoalteromonas* sp. SM9913: cloning, expression, characterization and function analysis of the C-terminal PPC domains

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Abstract Only a few cold-adapted halophilic proteases have been reported. Here, the gene *mcp03* encoding a cold-adapted halophilic protease MCP-03 was cloned from deep-sea psychrotolerant bacterium *Pseudoalteromonas* sp. SM9913, which contains a 2,130-bp ORF encoding a novel subtilase precursor. The recombinant MCP-03, expressed in *Escherichia coli* BL21 and purified from fermented broth, is a multi-domain protein with a catalytic domain and two PPC domains. Compared to mesophilic subtilisin Carlsberg, MCP-03 had characteristics of a typical cold-adapted enzyme (e.g., higher activity at low temperatures, lower optimum temperature and higher thermolability). MCP-03 also exhibited good halophilic ability with maximal activity at 3 M NaCl/KCl and good stability in 3 M NaCl. Deletion mutagenesis showed that the C-terminal PPC domains were unnecessary for enzyme secretion but had an inhibitory effect on MCP-03 catalytic efficiency and were essential for keeping MCP-03 thermostable.

Keywords MCP-03 · Subtilase · Cold-adapted · Halophilic · *Pseudoalteromonas* sp. SM9913 · PPC domain · Deep sea

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Abbreviations

C-t	C-terminal
N-t	N-terminal
PPC domain	Bacterial pre-peptidase C-terminal domain
PKD domain	Polycystic kidney disease domain
FAAF	*N*-Succinyl-Phe-Ala-Ala-Phe-*p*-nitroanilide
AAPF	*N*-Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide
AAPR	*N*-Succinyl-Ala-Ala-Pro-Arg-*p*-nitroanilide
AAPL	*N*-Succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide
FVR	*N*-Benzoyl-Phe-Val-Arg-*p*-nitroanilide
AAPK	*N*-Succinyl-Ala-Ala-Pro-Lys-*p*-nitroanilide
AAA	*N*-Succinyl-Ala-Ala-Ala-*p*-nitroanilide
AAV	*N*-Succinyl-Ala-Ala-Val-*p*-nitroanilide
GGG	*N*-Succinyl-Gly-Gly-Gly-*p*-nitroanilide
GGF	*N*-Succinyl-Gly-Gly-Phe-*p*-nitroanilide

Introduction

The average depth of the oceans is about 3,800 m and almost 60% of the earth's surface is made up of the deep-sea floor (water depths greater than 2,000 m) (Brunnegard et al. 2004). The estimated total input of particulate organic nitrogen to the deep-sea sediment is 24–80 μmol/m^2/day and the recycling efficiency of sedimentary particulate organic nitrogen is 94% (Brunnegard et al. 2004). It has been shown that high proteolysis rate exists in deep-sea sediments (Bianchi et al. 2003; Huston and Deming 2002), suggesting that there are abundant

bacterial proteases. However, the bacterial proteases in deep-sea sediments are largely unknown. Although the proteases from some deep-sea *Pseudoalteromonas* (Chen et al. 2003, 2007; Xiong et al. 2007; Kurata et al. 2007) and *Pseudomonas* strains (Zeng et al. 2003) have been studied, more knowledge of the bacterial proteases involved in sedimentary organic nitrogen degradation is needed to understand the mechanism of organic nitrogen degradation and marine nitrogen recycling. In addition, since deep-sea bacterial proteases are relatively seldom exploited, their further study may find new proteases for future technological applications.

Based on catalytic type, proteases include aspartic, cysteine, metallo, serine, threonine and yet unclassified proteases (Barrett et al. 2004). Peptidase family S8, also known as the subtilisin family or subtilase family, is the second largest family of serine proteases. Subtilases are all characterized by a D/H/S catalytic triad and an alpha/beta-fold catalytic center containing seven-stranded parallel beta-sheets (Rawlings et al. 2006). Although subtilase precursors are usually mosaic proteins, the mature form of subtilases is usually mono-domain (Barrett et al. 2004; Siezen and Leunissen 1997). Some members from *Vibrio* and its close relatives are mosaic proteins and possess a special C-terminal (C-t) domain required for secretion, which is proteolytically removed upon extracellular activation (Barrett et al. 2004). However, it has been found that some mature subtilases are mosaic proteins with C-t domains, and the function of some C-t domains has been elucidated (Chen et al. 2007; Itoi et al. 2006; Tsujbo et al. 1996; Zhao et al. 2008). For example, the C-t domains of some subtilases with collagenolytic activity were able to bind collagen (Itoi et al. 2006; Zhao et al. 2008).

Pseudoalteromonas sp. SM9913 was isolated from deep-sea sediment as an efficient producer of extracellular proteases. We have been studying the extracellular proteolytic system of strain SM9913 to clarify the role of the strain and its proteases in sedimentary organic nitrogen degradation, the structure and function of the proteases and their adaptation to the deep-sea environment. The proteolytic system of strain SM9913 consists of at least three extracellular proteases (MCP-01, MCP-02 and MCP-03). MCP-01, the most abundant protease secreted by strain SM9913, is a cold-adapted protease (Chen et al. 2003). It is a new type of multi-domain subtilase (named deseasin) with a polycystic kidney disease (PKD) domain at its C-terminus, which can bind insoluble protein, such as collagen (Chen et al. 2007; Zhao et al. 2008). MCP-02 is a cold-adapted metalloprotease belonging to M4 family, and its cold adaptation mechanism was recently studied in detail (Xie et al. 2009). In this study, the gene encoding protease MCP-03 was cloned, sequenced and expressed in *E. coli*. The recombinant protease was purified and characterized, which showed that it is a cold-adapted multi-domain subtilase with high activity under extreme saline conditions. Moreover, the function of the C-t PPC domains of MCP-03 was studied by deletion mutagenesis.

Materials and methods

Cloning of the gene *mcp-03* encoding serine protease MCP-03

The genomic DNA of *Pseudoalteromonas* sp. SM9913 was prepared according to a previously described method (Chen et al. 2007). Two primers were designed according to the conserved sequences of the serine protease active sites (Maciver et al. 1994), and then a 565-bp DNA fragment was amplified by PCR from the genomic DNA of strain SM9913. With the 565-bp DNA fragment as a probe, one positive clone containing 83.5% of the total protease gene was observed by Southern blotting and colony hybridization using the method described by Taguchi et al (1995). Using TAIL PCR (Thermal Asymmetric Interlaced PCR) (Liu and Whittier 1995), the absent sequence of this gene and the 374 upstream base-pairs were cloned. Through assembly, a 2,504-bp sequence containing a 2,130-bp open reading frame (ORF) encoding MCP-03 was obtained. This gene, named *mcp-03*, was submitted to GenBank under Accession No. DQ422814.

Plasmids and mutagenesis

The expression plasmid pET22b-MCP03 was constructed by ligation of gene *mcp-03* into the *Eco*RI–*Xho*I restriction sites of the pET22b plasmid (Invitrogen). Using the pET22b-MCP03 plasmid as a template, the DNA fragments of progressive C-t truncation mutants, BQ1, BQ2, BQ3 and BQ4, were generated by PCR, respectively, using the method described by Pues et al (1997). The generated DNA fragments were subcloned into the vector pET22b for mutant expression.

Expression and purification

All the expression plasmids constructed above were transformed into *E. coli* BL21-(DE3) competent cells. All the proteins were expressed as C-terminally His_6-tagged proteins and purified with a His•Bind metal chelating column. To express active MCP-03 and the mutants from the transformed *E. coli* BL21, the transformed strain was grown in LB medium containing 50 μg/mL ampicillin at 37°C for 12 h. The culture was diluted 100-fold and grown

in LB-amp at 37°C to an $OD_{600} = 1.0$. Expression was induced with 0.25 mM IPTG with agitation at 150 rpm at 15°C. After 50 h (when the protease activity in the fermented broth peaked), the fermented broth was centrifuged at 10,000*g* at 4°C to remove the *E. coli* BL21 cells. The supernatant was incubated for another 4 h at 15°C to promote protease precursor maturation. The supernatant proteins were precipitated by 65% ammonium sulfate saturation. The dissolved proteins, after dialysis, were added to a His Bind metal chelating column (Novagen) and were eluted with 1 M imidazole. Proteins were pooled, and their purity was analyzed by SDS-PAGE using the Laemmli method (Laemmli 1970). All purification procedures were performed at 0–4°C.

Enzyme activity assay and characterization of MCP-03 and its mutants

The proteolytic activity of recombinant protease MCP-03 and its truncated mutants was assayed using synthetic peptide *N*-Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (AAPF, 0.5 mg/mL) as substrate at 45°C for 10 min with Peek's method (Peek et al. 1993). The optimum pH of MCP-03 was determined using a previously described method (Chen et al. 2003), and the optimum temperature was determined by monitoring MCP-03 activity over 10 min at optimum pH between 0 and 60°C. The thermostability was studied by incubating the enzyme at 50°C and the residual enzyme activity was measured every 5 min. The effects of phenylmethylsulfonyl flouride (PMSF, 10 mM), urea (3 M and 6 M), SDS (1%, w/v), Triton X-100 (1%, v/v), dithiotreitol (10 mM), NaCl and KCl (0–5 M) on MCP-03 activity were investigated by measuring MCP-03 activity at 45°C after MCP-03 was incubated with every agent in 50 mM tris–HCl (pH 8.0) containing 10 mM $CaCl_2$ at 0°C for 30 min. To analyze the substrate specificity of MCP-03, the ability to hydrolyze 10 synthetic peptides (0.5 mg/mL), FAAF (*N*-Succinyl-Phe-Ala-Ala-Phe-*p*-nitroanilide, the following peptides were abbreviated in the same way), AAPF, AAPR, AAPL, FVR, AAPK, AAA, AAV, GGG and GGF, was measured at 45°C using Peek's method (Peek et al. 1993). K_m values of MCP-03 and its mutants were determined by Lineweaver–Burk plots, which were made by linear regression with initial rates between 0 and 1 mg/mL of AAPF at 45°C. k_{cat} values of MCP-03 and its mutants were calculated with the formula $k_{cat} = V/[E]$, where V is the reaction rate measured at 45°C and [E] is the enzyme concentration in the reaction mixture. Protein concentration was assessed using the Bradford method (Bradford 1976). The N-terminal (N-t) amino acid sequence of the active MCP-03 protease was determined as previously described (Chen et al. 2007).

Results

Cloning and sequence analysis of gene *mcp-03* encoding protease MCP-03

The gene *mcp-03* encoding protease MCP-03 from *Ps.* sp. SM9913 was cloned and sequenced. As shown in Fig. 1, the gene contains a single complete ORF composed of 2,130 bp with an initiation codon of ATG and a termination codon of TAA. A possible TATA-like promoter site (5′-TATAAA-3′) is located 14 bp upstream of the initiation codon for transcription. The gene encodes a polypeptide consisting of 709 amino acids with a calculated molecular mass of 72.6 kDa. BLAST amino acid sequence similarity searches showed that *mcp-03* is translated as a subtilisin-like protease precursor.

The protease precursor deduced from *mcp-03* consists of four domains: a signal peptide sequence, an N-t prosequence, a catalytic domain and a C-t extension (Fig. 1). The first 27 N-terminal (N-t) amino acids is a signal peptide sequence identified by the SignalP program (Bendtsen et al. 2004). The five N-t amino acid residues of the purified enzyme were determined to be P1–F2–A3–T4–P5 by Edman degradation sequencing analysis. Based on the C-t sequence of the signal peptide and the N-t sequence of mature MCP-03 protease, the MCP-03 N-t prosequence is composed of 118 amino acid residues (N-118–K-1) and has high identity to the N-t prosequences of other subtilisins, especially in the N1 and N2 motifs that were speculated to be critical for nucleation of the folding process (Shinde et al. 1999). Therefore, the MCP-03 N-t prosequence may function as an intramolecular chaperone to guide correct folding, like in other subtilisins (Shinde et al. 1997, 1999). Amino acid sequence homology analysis of the MCP-03 catalytic domain (P1-S341) with other subtilisin-like proteases indicated that the three amino acid residues (D43, H102 and S280) that likely form the catalytic triad and their surrounding residues are fully conserved (data not shown). Even though MCP-03 has the highest identity (82%) and similar precursor structure with protease AprI secreted by the marine bacterium *Alteromonas* sp. strain O-7 (Tsujbo et al. 1996), the MCP-03 catalytic domain exhibits low identity (35%–44%) to other subtilisin family members, such as subtilisins BPN′ (44%) (Wells et al. 1983), subtilisins E (42%) (Stahl and Ferrari 1984), subtilisin Carlsberg (41%) (Jacobs et al. 1985) and the psychrophilic subtilisins, S41 (35%) (Davail et al. 1994) and S39 (36%) (Narinx et al. 1992). The C-t extension of MCP-03 precursor is characterized by a repeat of PPC domain (Bacterial Pre-peptidase C-terminal domain), PPC1 + PPC2, which are each composed of 85 amino acid residues. Two PPC domains show 96.4–97.6% and 87.3–92.9% similarity, respectively, to those in the C-t pro-region of

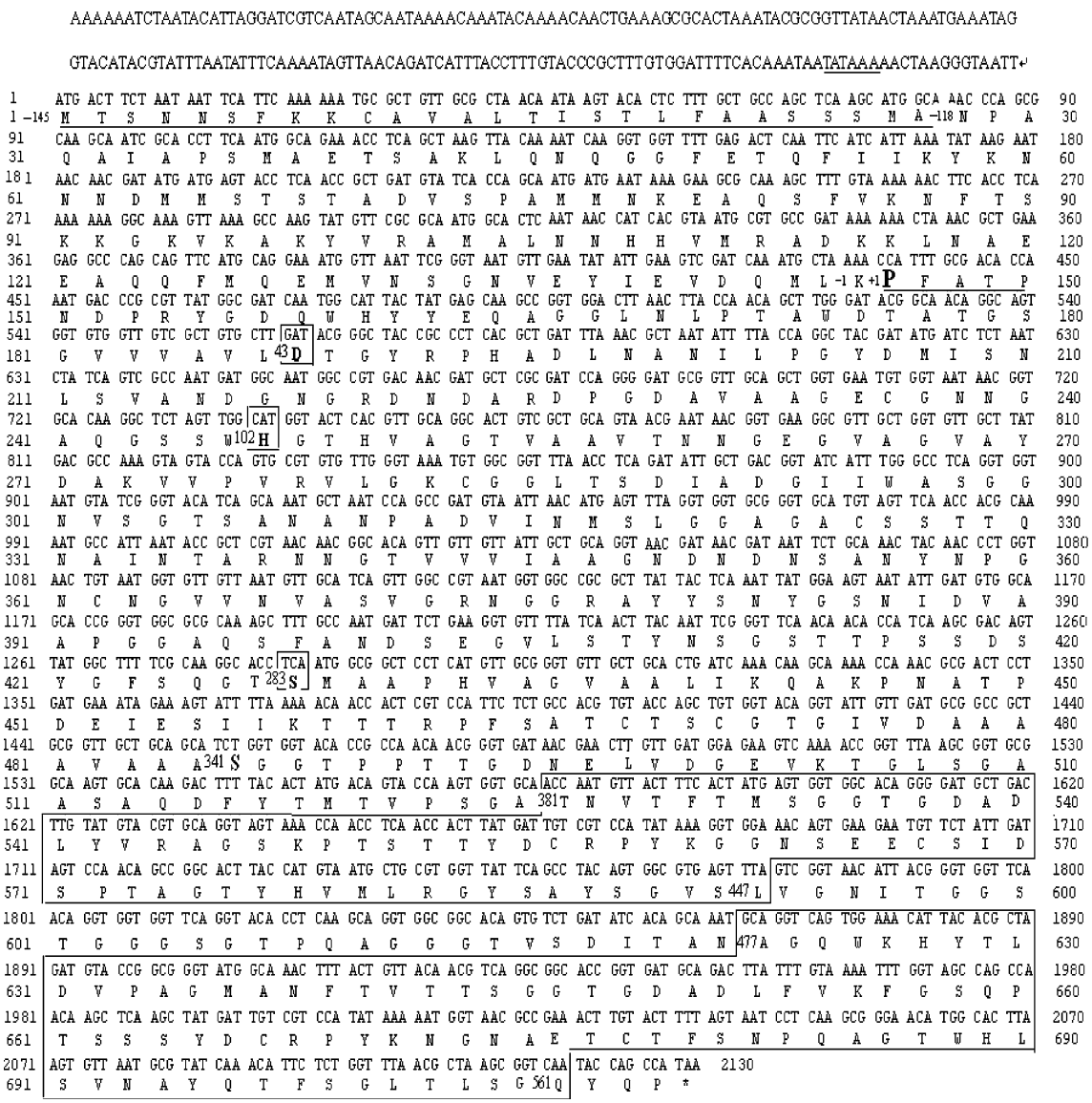

Fig. 1 Nucleotide and deduced amino acid sequences of the MCP-03 precursor. A possible TATA-like promoter site (5′-TATAAA-3′) located 14-bp upstream of the initiation codon. The first 27 amino acid residues underlined are the predicted signal peptide. The first amino acid residue of mature MCP-03 is written in bold and indicated by +1. Three amino acid residues—D43, H102 and S283—forming the catalytic triad are written in bold and boxed. The sequences of two C-t PPC domains are boxed, respectively

several known Gram-negative bacterial proteases in NCBI database (P70765, Q00971, Q9LCJ5 and Q60106).

Characterization of protease MCP-03

Mcp-03 was cloned into pET22b and expressed in *E. coli* BL21 (DE3). The active form of recombinant MCP-03 protease was purified from the fermented broth with a molecular weight of approximately 58 kDa analyzed by SDS-PAGE (Fig. S1). Based on its N-t sequence and molecular weight, active MCP-03 is composed of the catalytic domain and the two C-t PPC domains.

Since MCP-03 sequence analysis showed it is probably a subtilase of serine proteases, 10 synthetic substrates of subtilases and other serine proteases were selected to analyze MCP-03 specificity. The specific activities of MCP-03 to these substrates were measured and compared with those of subtilisin Carlsberg, the subtilase family archetype. As shown in Table 1, both enzymes very effectively hydrolyzed AAPF. AAPL, AAPK, AAPR, FAAF and FVR were hydrolyzed less effectively; and AAA, AAV, GGF and GGG had hydrolysis below the detection limit of our assay. These results indicated that MCP-03 has broad substrate specificity.

Table 1 Specific activities of MCP-03 and subtilisin Carlsberg toward synthetic substrates

Substrate	Specific activity of MCP-03 (U/mg)	Specific activity of subtilisin Carlsberg (U/mg)
AAPF	4,300	59,040[a]
AAPL	470	51,498[a]
FAAF	430	54,839
AAPK	345	8,165
AAPR	645	8,368
FVR	486	0[a]
AAA	0	9,812[a]
AAV	0	5,636[a]
GGF	0	12,223[a]
GGG	0	6,715[a]

The enzymatic activity was determined at pH 8, 45°C for MCP-03 and pH 10, 55°C for subtilisin Carlsberg. The experiment was performed in triplicate, and standard errors were within 5%

[a] These data are cited from our previous study (Chen et al. 2007)

With AAPF as a substrate, MCP-03 had the highest activity at 45°C and retained 14% of its maximal activity at 0°C. In contrast, subtilisin Carlsberg, a mesophilic subtilase, only had ~1% of its maximal activity at 0°C (Fig. 2a). As such, MCP-03 had higher relative activity at low temperatures than subtilisin Carlsberg. In addition, the optimal temperature of MCP-03 against AAPF was about 15°C lower than that of subtilisin Carlsberg. MCP-03 had high thermolability, in which activity dropped rapidly at 50°C (Fig. 2b). These MCP-03 results, compared with subtilisin Carlsberg, indicated that MCP-03 has cold-adapted enzyme characteristics. MCP-03 displayed an alkaline pH activity profile with an optimal pH of 8.0 (Fig. 2c), approximately the pH of seawater, indicating its adaptation to the sea environment.

MCP-03 exhibited the highest activity in 3 M NaCl/KCl and retained higher activity in 5 M NaCl than in 0 M NaCl (Fig. 2d). Moreover, MCP-03 was stable for at least 1 month at 4°C in 20 mM Tris–HCl (pH 8.0) containing 3 M NaCl and 10 mM $CaCl_2$ (data not shown). These results showed that MCP-03 is a halophilic protease.

The activity of MCP-03 was completely inhibited by 10 mM PMSF. It was sensitive to urea (3–6 M), 1% SDS and 1% Triton X-100, indicating that these protein denaturants could cause partial denaturation of MCP-03. As much as 10 mM dithiotreitol, a thiol-reducing agent, inhibited MCP-03 activity by 50% (Table 2).

Analysis of the MCP-03 C-t PPC domain function by deletion mutagenesis

In order to study the function of the C-t extension of MCP-03, an extensive C-t truncation analysis by deletion mutagenesis was undertaken. As shown in Fig. 3a, the mutant BQ1 was lacking the PPC2 domain at the C-terminus; BQ2 and BQ3 were lacking PPC2 and a part of PPC1 domain; BQ4 was lacking both PPC1 and PPC2. MCP-03 and the mutants, BQ1 to pBQ4, were expressed as C-terminally His_6-tagged proteins and purified directly from fermented broth (Fig. 3b), suggesting that the PPC domains in the C-t extension of MCP-03 are unnecessary for its secretion through the *E. coli* membrane. With AAPF as a substrate, the K_m values of the recombinant enzyme and all mutants were similar (Table 3). However, the PPC domains had inhibitory effects on MCP-03 catalytic efficiency, because the k_{cat}/K_m value of MCP-03, which has two PPC domains in its C terminus, was much lower than that of the mutants containing one or no PPC domain (Table 3). The thermal stability experiment revealed that MCP-03 had higher thermostability than any mutant at 50°C, and the thermostability of pBQ1 with one PPC domain was higher than any other mutant with a partial or no PPC domain (Table 3). These results suggested that the MCP-03 PPC domains may play a role in enzyme thermostability.

Discussion

Most deep-sea environments are extreme, influenced by low temperature, low nutrient concentration, high hydrostatic pressure, moderate salinity and fluidity. In order to adapt to such a harsh environment, bacterial proteases may have special structures and functions to carry out protein degradation. For example, the protease secreted by deep-sea bacterium *Pseudomonas* strain DY-A and protease MCP-01 from deep-sea strain SM9913 are cold-adapted enzymes (Zeng et al. 2003; Chen et al. 2003). Protease MCP-01 has a C-t PKD domain, which can bind to insoluble proteins to facilitate protein degradation by MCP-01 (Zhao et al. 2008). Exploration of the deep-sea proteases may not only find novel proteases with special structures and functions, but may also be helpful in understanding deep-sea nitrogen recycling.

In this study, the gene *mcp03* encoding protease MCP-03 from deep-sea cold-adapted bacterium *Pseudoalteromonas* sp. SM9913 was cloned and expressed in *E. coli*, and the active enzyme of recombinant MCP-03 was purified and characterized. Sequence analysis of MCP-03 showed that it is a novel subtilase. The recombinant MCP-03 is a multi-domain subtilase with two PPC domains at its C-terminus. Compared to mesophilic subtilisin Carlsberg, MCP-03 had typical characteristics of a cold-adapted enzyme including higher activity at low temperatures, lower optimum temperature and higher thermolability, indicating that it is a cold-adapted enzyme. Subtilisin Carlsberg is the archetype

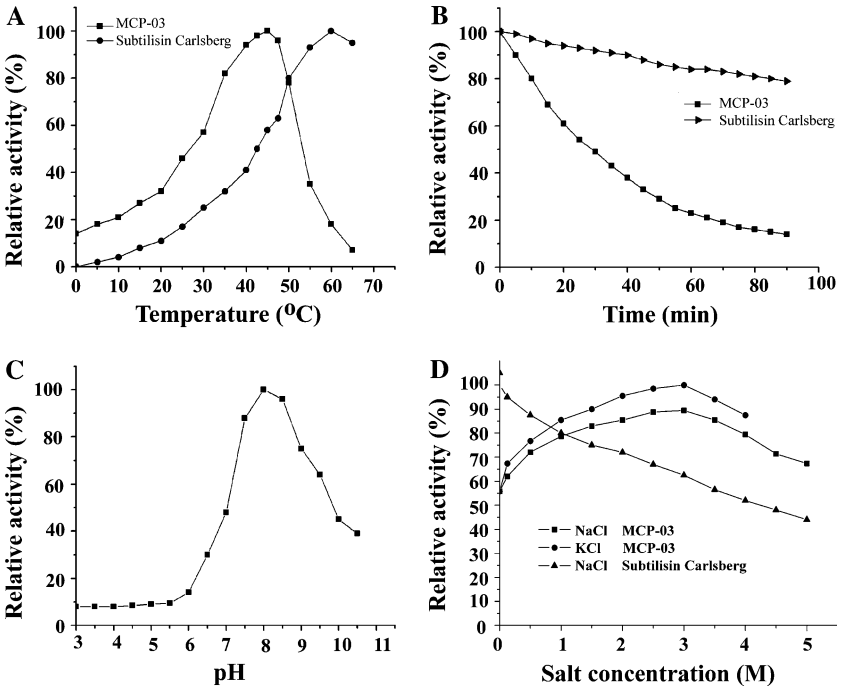

Fig. 2 **a** Effect of temperature on MCP-03 and subtilisin Carlsberg activity. Enzyme activities toward AAPF were measured at pH 8.0, 0–65°C. The specific activity of MCP-03 (4,300 μ/mg) at 45°C and the specific activity of subtilisin Carlsberg (63,484 μ/mg) at 60°C correspond to 100% activity, respectively. **b** Effect of temperature on the stability of MCP-03 and subtilisin Carlsberg. Enzymes were incubated at 50°C, and the residual activity toward AAPF was measured every 5 min at pH 8.0, 45°C for MCP-03 and 60°C for subtilisin Carlsberg. The specific activities of MCP-03 (4,300 μ/mg) and subtilisin Carlsberg (63,484 μ/mg) without incubation correspond to 100% activity, respectively. **c** Effect of pH on the activity of MCP-03. The activity of MCP-03 toward AAPF was measured at 45°C in broad pH buffers ranging from pH 3 to pH 11 as previously described (Chen et al. 2003). The specific activity of MCP-03 (4,300 μ/mg) at pH 8.0 corresponds to 100% activity. **d** Effect of NaCl and KCl on MCP-03 and subtilisin Carlsberg activity. The enzyme activity was measured with AAPF as substrate at 45°C for MCP-03 and 60°C for subtilisin Carlsberg in 20 mM Tris–HCl (pH 8.0) containing NaCl (or KCl) and 10 mM $CaCl_2$. The specific activity of MCP-03 (7,697 μ/mg) in 3 M NaCl and the specific activity of subtilisin Carlsberg (63,484 μ/mg) in 0 M NaCl correspond to 100% activity, respectively

Table 2 Effects of protease inhibitors, denaturing agents, detergents and thiol-reducing agents on the activity of protease MCP-03

Reagent	Reagent group	Final concentration	Relative activity (%)
Control			100[a]
PMSF	Inhibitor	10 mM	0
SDS	Detergent	1% (w/v)	33
Triton X-100	Detergent	1% (v/v)	53
Urea	Denaturing agent	3 M	45
Urea	Denaturing agent	6 M	20
Dithiothreitol	Reducing agent	10 mM	50

The protease was incubated with each chemical for 30 min at 0°C, and the enzyme activity was determined at pH 8, 45°C with 0.5 mg/mL AAPF as a substrate. The experiment was performed in triplicate, and standard errors were within 5%

[a] The specific activity of MCP-03 without any of the listed chemicals corresponds to 100% activity

of subtilase family (Barrett et al. 2004). Most subtilases are nonspecific peptidases with a preference for an aromatic amino acid residue at the P1 position (Siezen and Leunissen 1997). MCP-03 has broad substrate specificity. The broad substrate specificity of MCP-03 is beneficial for strain SM9913 to adequately utilize the surrounding deep-sea proteins. MCP-03 displayed alkaline pH activity profile (pH 7.0 to 9.0) with an optimal pH of 8.0, approximately the pH of the seawater. Moreover, MCP-03 is a halophilic protease with the highest activity in 3 M NaCl/KCl. These results suggest adaptation of MCP-03 to the alkaline, cold, saline and low-nutrient deep-sea environment. Although many halophilic proteases from archaea and bacteria have been reported (Mellado et al. 2005), only a few are cold-adapted halophilic proteases (Xiong et al. 2007). These cold-adapted halophilic proteases may aid in processing of saline food, such as seafood.

The PPC domain is found in some members of metalloprotease families M4, M9 and M28 as well as serine protease family S8 (Barrett et al. 2004). The PPC domains are usually cleaved after secretion but prior to protease activation (Yeats et al. 2003). There are few mature proteases containing PPC domains. While their actual function is not clear, they may aid secretion/

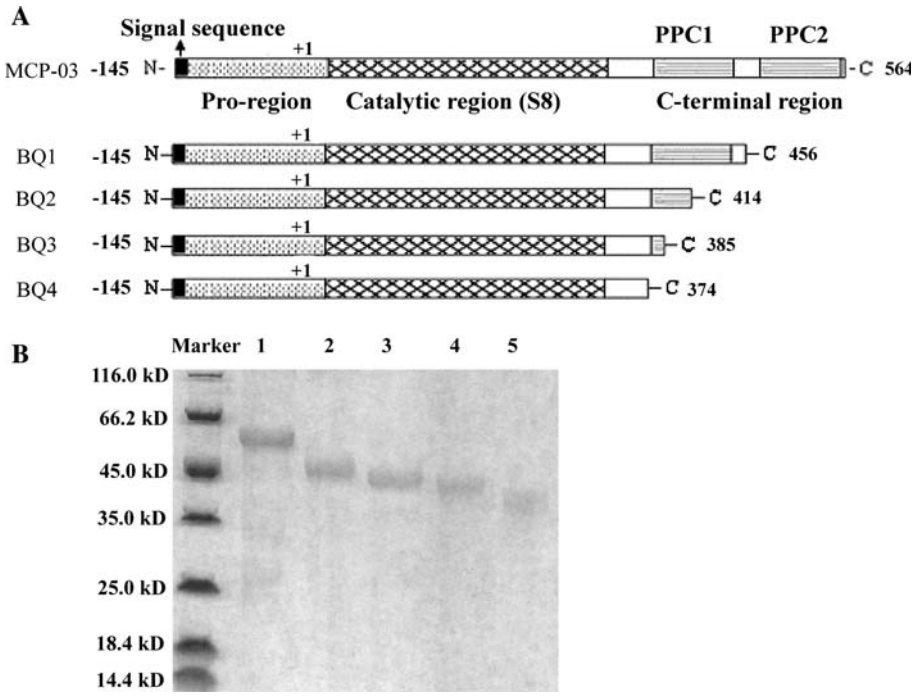

Fig. 3 **a** Schematic diagram of MCP-03 and its C-terminal truncated mutants. The number at the left and the right of the schematic diagram represents the location of the first and the last amino acid residue of the MCP-03 precursor sequence shown in Fig. S1. **b** The purified active enzymes of MCP-03 and its C-terminal truncated mutants of MCP-03 analyzed by 12.5% SDS-PAGE. *Lane 1*, MCP-03; *lane 2*, BQ1; *lane 3*, BQ2; *lane 4*, BQ3; *lane 5*, BQ4

Table 3 Characteristics of wild-type and truncated mutants of protease MCP-03

Protease	Number of amino acid residues	Deleted C-terminal residues (total no.)	Precursor mass (kDa)	Mature protease mass (kDa)	Half time at 50°C[a] (min)	K_m^b (mM)	k_{cat}^c (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
MCP-03	709	0	72.6	56.6	30	0.068	23.5	346
BQ1	601	108	61.5	45.5	25	0.059	175.7	2,978
BQ2	559	150	57.3	41.3	20	0.063	234.3	3,719
BQ3	530	179	54.3	38.3	20	0.062	243.5	3,927
BQ4	519	189	53.3	37.3	20	0.060	233.7	3,895

[a] MCP-03 and the mutants were incubated at 50°C, and the residual enzyme activity was measured at indicated time intervals (Fig. S2). Half-time was the time that an enzyme took to lose a half of its activity at 50°C

[b] K_m values of MCP-03 and the mutants were determined by Lineweaver–Burk plots, which were made by linear regression with initial rates determined between 0 and 1 mg/mL of AAPF at 45°C (Fig. S3)

[c] k_{cat} values of MCP-03 and the mutants were calculated with the formula $k_{cat} = V/[E]$, where V is the reaction rate measured at 45°C and [E] is the enzyme concentration in the reaction mixture

localization or inhibit the protease until needed (Yeats et al. 2003). The PPC domain of protease XCEXPR is reportedly required for secretion through the *E. coli* JM109 outer membrane (Liu et al. 1990). Proteases AprI and AprII with C-t PPC domains show lower activity than the matured enzymes without PPC domains (Tsujbo et al. 1996). The homologous C-t PPC domain of *V. vulnificus* metalloprotease is essential for efficient attachment to insoluble protein substrates and erythrocyte membranes (Miyoshi et al. 1997). In this paper, the mature recombinant MCP-03 enzyme has been shown to have a catalytic domain and two PPC domains at its C-terminus. In order to study the effect of these PPC domains on MCP-03, a series of deletion mutants were designed and expressed. The MCP-03 C-t PPC domains are unnecessary for secretion through the *E. coli* membrane, similar to *V. vulnificus* metalloprotease (Miyoshi et al. 1997). Moreover, the MCP-03 C-t PPC domains had inhibitory effect on catalytic efficiency since the mutant without any PPC had higher catalytic efficiency than MCP-03 and the mutant with one PPC. This is consistent with the effect of PPC on the activity of proteases AprI and AprII (Tsujbo et al. 1996). In addition, the MCP-03 PPC domains might aid enzyme thermostability because MCP-03 and the mutant with one PPC are more thermostable than the mutants lacking PPC.

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