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Note

Cloning and Characterization of Extracellular Metal Protease Gene of the Algicidal Marine Bacterium *Pseudoalteromonas* sp. Strain A28

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The gene (*empI*) encoding an extracellular metal protease was isolated from a *Pseudoalteromonas* sp. strain A28 DNA library. The recombinant EmpI protein was expressed in *E. coli* and purified. Paper-disk assays showed that the purified protease had potent algicidal activity. A skim milk-polyacrylamide gel electrophoresis protease assay showed that the 38-kDa band of protease activity, which co-migrated with purified EmpI and was sensitive to 1,10-phenathroline, was detected in the extracellular supernatant of A28.

Key words: *Pseudoalteromonas*; algicidal marine bacterium; extracellular metal protease; red tide algae

Pseudoalteromonas sp. strain A28 was isolated from a seawater sample from the Ariake Sea of Japan where the marine diatom *Skeletonema costatum* occasionally produces undesirable blooms.[1,2] This organism was able to kill *S. costatum*, *Thalassiosira* sp., *Eucampia zodiacs*, and *Chattonella antiqua*. The culture supernatant of A28 showed potent algicidal activity and physical contact of the bacteria with cells was not required for algicidal effects. A28 excretes several proteins including proteases and DNase. One of the extracellular proteins, protease I, had potent algicidal activity.[2] Protease I was a monomeric protein with a molecular mass of 50 kDa. The proteolytic activity of protease I was strongly inhibited by serine protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP). Thus, *Pseudoalteromonas* sp. strain A28 produces an extracellular serine protease that is responsible for the algicidal activity of this marine bacterium. Addition of PMSF diminished algicidal activity of the A28 culture supernatant, however, it did not result in a complete loss of algicidal activity. This result prompted us to search for another algicidal substance of A28 other than protease I. Some pathogenic bacteria, such as *Pseudomonas aeruginosa* and *Vibrio vulnificuns*, produce more than one protease that have been suggested to be required for the tissue destruction.[3–6] A28 may also produce multiple proteases that are involved in its algicidal activity. In this paper, we describe cloning of the *Pseudolateromonas* sp. strain A28 gene encoding a metal protease. We also show that the gene product had algicidal effects on *S. costatum*.

Pseudoalteromonas sp. strain A28, *Escherichia coli*, and *S. costatum* NIES-324 were cultivated as described previously.[1] A *Pseudoalteromonas* sp. strain A28 genomic library was constructed by ligating 4- to 7-kb *Sau*3AI partially cleaved DNA fragments to the *Bam*HI site of pUC118.[7] The ligated DNA fragments were used to transform the host strain *E. coli* HB101.[8] Transformants were screened for protease activity by selection on LB-agar plates containing 1% Bacto-skim milk (Difco) and 100 μg ampicillin /ml. From a screen of approx. 30,000 colonies, three clones were selected which had zones of clearing. All of the recombinant plasmids were shown to contain overlapping DNA insert fragments. A single plasmid (pPEMP01) was selected for further analysis. A restriction map of pPEMP01 was constructed (Fig. 1). The 5.2-kb insert of pPEMP01 was digested with various restriction enzymes, and the fragments were subcloned into pUC118 to make pPEMP01.1, pPEMP01.2, and pPEMP01.3 (Fig. 1). *E. coli* HB101 (pPEMP01.1) formed a zone of clearing on L-agar with added skim milk and ampicillin. Neither *E. coli* HB101 (pPEMP01.2) nor *E. coli* HB101 (pPEMP01.3) showed proteolytic halos. Thus, the gene encoding the protease was localized to a 2.8-kb *Sau*3AI-*Pst*I fragment of pPEMP01.

Nucleotide sequence analysis of the pPEMP01.1 insert showed that the 2.8-kb *Sau*3AI-*Pst*I fragment contained a potential open reading frame (*empI*)

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Abbreviations: DFP, diisopropyl fluorophosphate; EGTA, ethylene glycol bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride

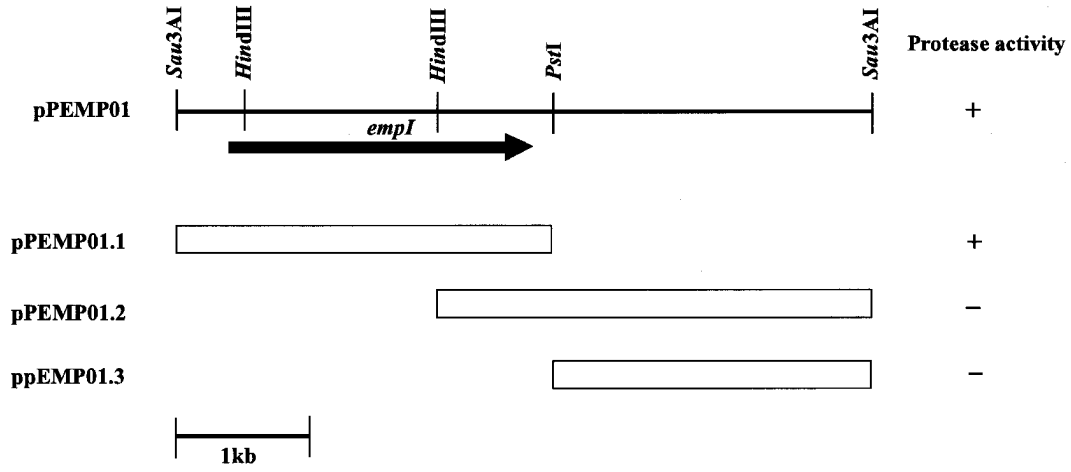

Fig. 1. Restriction Map of Plasmid pPEMP01 Containing the 5.2-kb *Sau*3AI Fragment of the *Pseudoalteromonas* sp. Strain A28 Chromosomal DNA and Its Subclones.

The location and orientation of *empI* are indicated by a horizontal arrow. Open bars indicate the DNA fragments subcloned into pUC118. The right part summarizes whether the plasmid enabled HB101 to form zones of clearing around its colonies on LB-plates supplemented with skim milk and ampicillin. The nucleotide sequence of the 2.8-kb *Sau*3AI-*Pst*I fragment of pPEP01.1 has been deposited in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases under the accession number AB070236.

(Fig. 1). The *empI* gene encoded a putative protein of 731 amino acids with a molecular weight of 77,784. A computer-assisted sequence similarity search revealed that a 304-amino-acid EmpI protein starting with Ala-207 showed 61.2, 63.0, and 64.4% identity with the mature *P. aeruginosa* elastase,[9] the *V. vulnificus* metal protease,[10] and the *V. proteolyticus* vibriolysin,[11] respectively. These proteases are zinc metalloproteases. It is interesting to note that the deduced C-terminal amino acid sequence of EmpI has significant similarity to the C-terminal region of the serine protease (AprI) of the marine bacterium *Alteromonas* sp. strain O-7, which undergoes C-terminal processing.[12] The C-terminal 219 amino acids of AprI and EmpI shared 48.4% amino acid identity. Like AprI, the two repeated amino acid sequences were found in the C-terminal region of EmpI. It was supposed that the degradation of the C-terminal proregion is essential for the formation of a more stable and active conformation of AprI.[12]

To further characterize the protease encoded by the *empI* gene, the protease was purified to homogeneity from the culture supernatant of *E. coli* HB101 (pPEMP01.1). *E. coli* HB101(pPEMP01.1) cultures were grown for 5 h at 28°C in LB medium containing 200 μg/ml of ampicillin, and the cells were harvested by centrifugation at 6,500 $\times g$ for 15 min. The proteins were precipitated by adding solid ammonium sulfate to 80% saturation to the culture supernatant in the ice bath. The precipitate was redissolved in TM buffer (20 mM Tris-HCl (pH 7.8), 2 mM $MgCl_2$) and put on an anion-exchange column (Poros HQ/M, 4.6 $\times$ 100 mm; PerSeptive Biosystems Inc., Framingham, Mass.). Proteins were eluted with a linear NaCl gradient of 0 to 1 M in TM buffer. Fractions with high protease activities were collected and dialyzed against 50 mM phosphate buffer (pH 7.0) containing 1 M ammonium sulfate. Protease activity was measured as azocasein (Sigma) hydrolytic activity as described previously.[2] The dialyzed sample was then put on a hydrophobic interaction column (Poros PH, 4.6 $\times$ 100 mm; PerSeptive Biosysytems Inc.). The column was developed with a linear gradient of 1 to 0 M ammonium sulfate. Active fractions were pooled and concentrated by ultrafiltration. Then, preparative native electrophoresis was done using a Mini Prep Cell (Bio-Rad) as described previously.[2] By SDS-PAGE, the molecular mass of the EmpI protease was estimated as 38 kDa (Fig. 2A). Since the molecular mass of the EmpI protease was also estimated as 38 kDa by gel filtration, the EmpI protease should be a monomer (data not shown). The N-terminal amino acid sequence of the purified protein was found to be Ala-Ser-Ala-Thr-Gly-Pro-Gly-Gly-Asn-Leu. The amino acid sequence for the N-terminal sequence was located within the 525-amino-acid polypeptide starting at Ala-207 and is in perfect agreement with all 10 sequence amino acids. The 525-amino-acid polypeptide is calculated to be 56 kDa, which is substantially larger than the 38 kDa of the purified EmpI protease. These results suggest that EmpI undergoes a removal of an 18-kDa polypeptide from its carboxy terminus. The optimum temperature and pH for proteolytic activity were 50°C and pH 8.6, respectively. Enzyme inhibitors were tested for the ability to block the hydrolysis of azocasein. EmpI was insensitive to PMSF (1 mM) and DFP (1 mM), suggesting that it is not a serine protease. EDTA (1 mM), EGTA (1 mM), and 1,10-phenanthroline (1 mM) decreased its activity by 48, 67, and 97%, respectively. These results suggest that the purified protease is a metal protease. The activity of A28 pro-

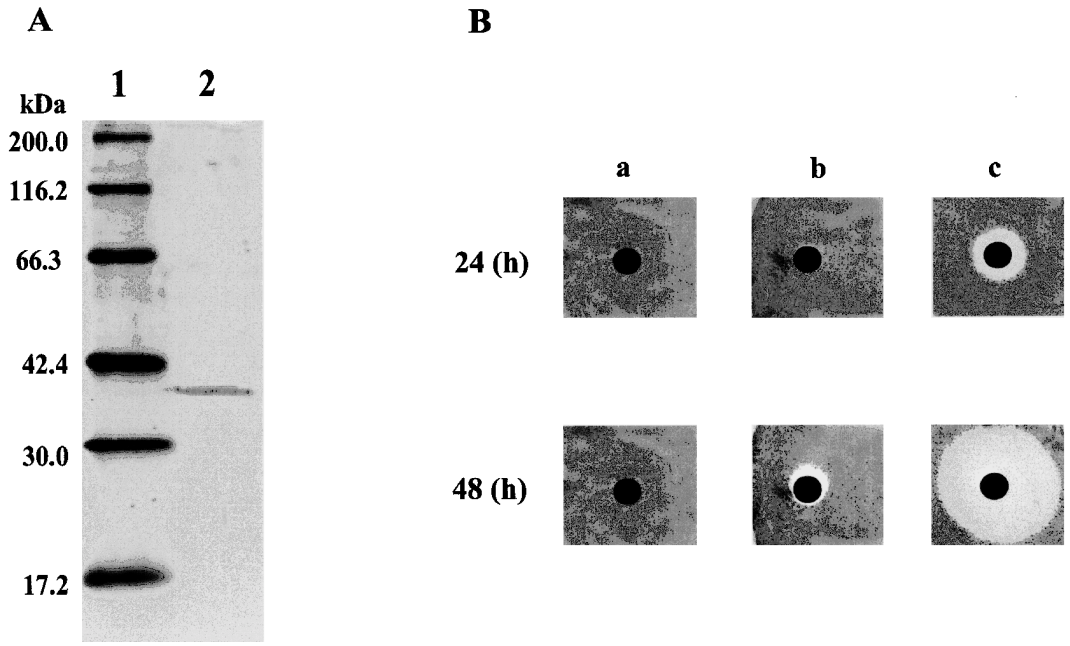

Fig. 2. SDS-PAGE Analysis (A) and Detection of Algicidal Activity (B) of Purified EmpI Protease from the Culture Supernatant of *E. coli* HB101 (pPEMP01.1).

A. The pooled sample was electrophoresed and silver stained. Lane 1, molecular mass markers; lane 2, EmpI-rich fraction from preparative native-protein gel electrophoresis. B. Algicidal activity was detected as described previously.[2] Zones of clearing around paper disks indicate the lysis of the diatom *S. costatum* strain NIES-324. Plates were photographed after 24 and 48 h of incubation at 20°C. a, TM buffer (20 μl); b, EmpI-rich fraction from preparative native-protein gel electrophoresis (2.4 μg of protein [0.16U]); c, EmpI-rich fraction from preparative native-protein gel electrophoresis (4.8 μg of protein [0.32U]).

tease I was completely inhibited by PMSF and DFP.[2] In addition, the N-terminal amino acid sequence of the purified A28 protease I (Ala-Thr-Pro-Asn-Asp-Pro) was not found in the deduced amino acid sequence of the *empI* gene. Therefore, EmpI is evidently a distinct protease from protease I.

The purified EmpI protease had strong algicidal activity in paper disk assays[2] on agar plates seeded with *S. costatum* (Fig. 2B). When 4.8 μg of EmpI (0.32U) was applied to a paper disk placed on a lawn of *S. costatum* cells, a clear zone was detected around the paper disk after 24 h of incubation. The zone of clearing was 17 mm in diameter. When 2.4 μg of EmpI (0.16U) was added to a paper disk, a distinct zone of clearing was not detected after 24 h of incubation. However, a clear zone (15 mm in diameter) appeared around the paper disk after 48 h of incubation. Since clear zones have been detected after 24 h of incubation by the application of 0.8 μg of A28 protease I (0.16U),[2] EmpI was 6-fold less algicidal to *S. costatum* than A28 protease I on the basis of the mass of proteins. Since the EmpI protease undergoes a removal of an 18-kDa polypeptide from its carboxy terminus, the 18-kDa carboxy terminal region is not required for algicidal activity.

To investigate whether *Pseudoalteromonas* sp. strain A28 produces EmpI protease or not, the extracellular supernatant of A28 was analyzed using skim-milk-SDS-PAGE gels (Fig. 3). Five major bands (117, 75, 59, 50, and 47 kDa) and three minor

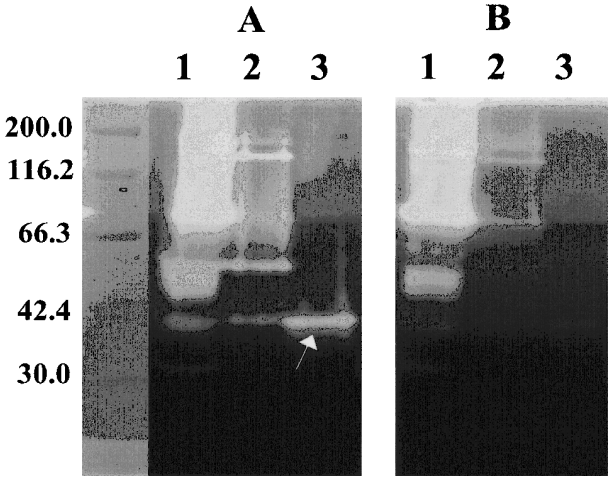

Fig. 3. Proteolytic Analysis of the Extracellular Supernatant Proteins from *Pseudoalteromonas* sp. Strain A28 and Purified EmpI Protease with Skim-Milk-SDS-PAGE.

Protease activity of separated proteins in a SDS-polyacrylamide gel was detected by copolymerizing 0.5% skim milk (Difco) in the polyacrylamide matrix. After electrophoresis, the gel was soaked in 2.5% Triton X-100 for 30 min at 4°C, incubated at 37°C for 1 h in 50 mM Tris-HCl buffer (pH 7.8) (A) or in 50 mM Tris-HCl buffer containing 5 mM 1,10-phenathroline (B), and then fixed and stained with 0.5% Coomassie Brilliant Blue R in ethanol-acetic acid-water (2:1:7, v/v/v). After the gel was destained with 10% (v/v) acetic acid-20% (v/v) ethanol, protease activity was detected by measuring zones of clearing in the SDS-skim milk-polyacrylamide gel. Lane 1, extracellular supernatant of A28; lane 2, extracellular supernatant of A28 treated with 1 mM PMSF before electrophoresis; lane 3, purified EmpI protease.

bands (130, 64, and 38 kDa) of protease activity were present in the supernatant fraction. When extracellular supernatant was treated with 1 mM PMSF before electrophoresis, the major bands of protease activity disappeared. The 38-kDa band co-migrated with the purified EmpI protease. The 38-kDa band was resistant to PMSF, while this band was not detected when gels were treated with 1,10-phenanthroline immediately after electrophoresis. These results suggest that the 38-kDa band in extracellular supernatant of A28 corresponds to EmpI.

In the previous study, we reported that A28 produced two serine proteases, protease I and protease II.[2)] Unlike protease I, protease II, with a molecular mass of 75 kDa, did not show any algicidal activity. Interestingly, some commercially available proteases, including trypsin, pepsin, subtilisin, and pronase, were examined for their algicidal activities, but none of them showed algicidal activity.[2)] In this study, we demonstrated that EmpI protease also had potent algicidal activity. To discover the molecular mechanisms underlying algicidal activity of protease I and EmpI, nucleotide sequence comparison of the protease I, protease II, and *empI* genes would be useful. We are now cloning the genes encoding *Pseudoalteromonas* sp. strain A28 protease I and protease II.

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