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ORIGINAL PAPER

Characterization of proteolytic bacteria from the Aleutian deep-sea and their proteases

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Abstract Six deep-sea proteolytic bacteria taken from Aleutian margin sediments were screened; one of them produced a cold-adapted neutral halophilic protease. These bacteria belong to *Pseudoalteromonas* spp., which were identified by the 16S rDNA sequence. Of the six proteases produced, two were neutral cold-adapted proteases that showed their optimal activity at pH 7–8 and at temperature close to 35°C, and the other four were alkaline proteases that showed their optimal activity at pH 9 and at temperature of 40–45°C. The neutral cold-adapted protease E1 showed its optimal activity at a sodium chloride concentration of 2 M, whereas the activity of the other five proteases decreased at elevated sodium chloride concentrations. Protease E1 was purified to electrophoretic homogeneity and its molecular mass was 34 kDa, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The molecular weight of protease E1 was determined to be 32,411 Da by mass spectrometric analysis. Phenylmethyl sulfonylfluoride (PMSF) did not inhibit the activity of this protease, whereas it was partially inhibited by ethylenediaminetetra-acetic acid sodium salt (EDTA-Na). De novo amino acid sequencing proved protease E1 to be a novel protein.

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Introduction

The deep-sea environment is characterized by a high hydrostatic pressure, moderate salinity, and low temperature. Microorganisms that live in the deep sea have presumably developed unusual strategies to enable themselves to adapt and thrive in such a harsh environment, and their metabolism may vary greatly from that of terrestrial microbes [9]. Cold-adapted psychrophilic and psychrotolerant microbes from the deep sea have been widely studied [7, 15, 25]. The degradation of organic matter and element cycles in the deep-sea environment rely on the capacity of enzymes to operate at ambient temperatures [8, 15]. A few cold-adapted proteases from bacteria have been found in perennially cold habitats, such as the polar regions or deep-sea sediments [7, 15, 25].

Bacteria produce most industrially used proteases, most of which have an optimum pH that falls within the neutral or alkaline pH ranges. By definition, neutral proteases are active in the pH range 5–8, whereas alkaline proteases are mostly active in the pH range 8–12 [5, 16]. The neutral bacterial proteases usually have a relatively low thermotolerance compared to alkaline proteases [16]. Cold-adapted or low thermotolerant enzymatic properties are advantageous for waste decomposition in cold environments, for food processing to limit hydrolysis, for food preservation, and for processes that require the rapid inactivation of enzymatic reactions [7, 15]. Neutral proteases also generate less bitterness in hydrolyzed food proteins, and are

more valuable for use in the food industry, such as in the fermentation of fish or soy sauce [6, 16, 18].

A few halotolerant proteases have been isolated and characterized from saline or similar environments [4, 6, 18, 19]. Halotolerant or halophilic enzymes exhibit some unique structural and biochemical characteristics, but it is not yet understood what stabilizes halophilic enzymes under extreme conditions of high salinity [13, 17]. Furthermore, halophilic enzymes may maintain their biocatalytic properties in organic or nonaqueous media, as the high salt concentration reduces water activity in principle [13]. The absence of water often results in new enzymatic reactions, such as a better thermostable performance, an increased physical rigidity, and an enzyme "molecular memory" in organic solvents [10]. Some enzyme-catalyzed reactions in organic solvents have already been commercialized [10]. Halophlic enzymes are an interesting scientific research topic not only because of their novel characteristics, but also because of their potential application in situations that require tolerance of a high salt concentration or low water activity such as in the antifouling coating and paint industry [24].

This study focuses on cold-adapted proteases that are produced by deep-sea bacteria obtained from the Aleutian margin. A neutral halophilic protease was purified and some of its biochemical and catalytic properties were described. The enzymatic properties and the BLAST results of the de novo amino acid sequences show that this protease is an unpublished novel protein.

Materials and methods

Sampling and isolation of protease-producing bacterial strains

Sediment samples were collected in July 2004 from three locations in the area of the Aleutian margin in the Gulf of Alaska in the Pacific Ocean (strains D1-001, D2-005, and D3-103 were collected from 53°27′N, 163°22′W at a depth of 4,240 m; strain D4-001 was collected from 53°30′N, 163°27′W at a depth of 3,283 m; strains D12-004 and D12-006 were collected from 53°30′N, 163°26′W at a depth of 3,310 m) by using the submarine JASON II. The sediments were kept at 0°C, transported to the laboratory, and stored at 4°C before processing. One gram of sediment was resuspended in 10 ml of filtered seawater (0.22-μm filter), and 200 μl of supernatant was plated on 2,216 marine agar (Difco Laboratories) and incubated at 4°C for 1 week. The bacterial cultures that were isolated from the sediments were screened for protease production on a skimmed milk agar medium that contained 2% of skimmed milk powder and 1% of tryptone (w/v). Colonies that showed a clear zone of proteolytic activity were selected and identified as containing protease-producing bacteria, and were maintained on 2,216 marine agar plates. Unless otherwise indicated, the reagents were purchased from Sigma-Aldrich Inc., USA.

Bacterial cultivation and taxonomic identification

The purified protease-producing bacterial strains were grown on 2,216 marine agar plates at room temperature (25°C), and separate single colonies were selected for PCR amplification. The 16S rDNA gene was amplified by the forward primer 355F (5′-ACTCCTACGGGAGGCAGC-3′) and the reverse primer 1055R (5′-CACGAGCTGACGACAGCCAT-3′) for the five bacteria (D1-001, D2-005, D3-103, D4-001, and D12-004): the forward primer 355F (5′-ACTCCTACGGGAGGCAGC-3′) and the reverse primer 1492R (5′-GGYTACCTTGTTACGACTT-3′) for strain D12-004. The primers were purchased from Invitrogen Life Technologies Inc, USA. The amplification procedure included 5 min at 94°C followed by 30 cycles of 50 s at 94°C, 60 s at 58°C, and 60 s at 72°C. In the last cycle, the 72°C step was extended for 10 min, and the samples were finally cooled down to 4°C. The PCR products were purified and sequenced with both the forward and the reverse primers [1]. The 16S rDNA sequence of strain D12-004 (*Pseudoalteromonas issachenkonii* UST041101-043) was deposited in GenBank as the code DQ178021. The homologies of the resulting sequences were searched for using the BLAST program that is available from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/). The six strains were individually grown in a 250 ml shake flask (100 rpm) with 100 ml of 2,216 marine broth at room temperature (25°C), and the cells were separated from the medium by centrifugation (8,000 rpm, 15 min). The spent culture media were collected for the characterization of protease enzymatic properties.

Protease purification

Protease purification followed the method described by Xiong et al. [22]. Briefly, the strain D12-004 was grown in a 2-l shake flask with 2,216 marine broth, and the cells were separated from the medium by centrifugation. Ammonium sulfate was added to the supernatant to achieve 80% saturation, and the spent culture

medium was then centrifuged and the precipitate dissolved into 25% saturated ammonium sulfate in 50 mM of Tris–HCl (pH 7.5). The sample was applied to a Phenyl Sepharose column (Amersham Pharmacia Biotech, Sweden) that had been pre-equilibrated with 25% saturated ammonium sulfate in 50 mM of Tris–HCl buffer (pH 7.5). The column was eluted with a linear gradient of 25–0% saturated ammonium sulfate. The active fractions were pooled and concentrated by ultrafiltration through a 10-kDa membrane (PM 10, Millipore) to one-tenth of the original volume, and 20 mM of citrate–phosphate buffer (pH 6.0) was added to achieve the original volume. The concentrated sample was applied to a DEAE Sepharose (Amersham Pharmacia Biotech) column for ion-change chromatography. The column was then eluted with a linear gradient of 0–1 M sodium chloride solution with 20 mM of citrate–phosphate buffer (pH 6.0). The fractions that showed protease activity were pooled and concentrated by ultrafiltration (Centriplus 30, Amicon, USA), and the purified enzyme was stored at −20°C in 20 mM ammonium acetate buffer (pH 6.0).

Protein identification

The purity of the enzyme was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out using 12% polyacrylamide gels according to the standard protocol [11]. The molecular weight of the enzyme was estimated using a standard protein marker (Catalog 10748-010, Invitrogen). Protein concentrations were determined using the Lowry method with bovine serum albumin (A-2153, Sigma) as standard protein. The molecular weight of the purified protease was determined by ESI mass spectrometry [22]. The de novo protein sequencing was carried out by the mass spectrometry facility at the Hong Kong University of Science and Technology in Hong Kong. Briefly, the protein (0.5 μg) was separated by SDS-PAGE, and the target protein band was then cut out for sequencing. The protein was reduced and then reacted with iodoacetimide before fragmentation using trypsin. After overnight digestion, the peptides were extracted and then dried. ZipTip was used to desalt the peptide sample before the sample underwent mass spectrometry analysis. De novo sequencing results were carried out by reading the MS/MS spectrum.

Protease activity assay

Protease activity was measured by assaying the absorbance of the liberated amino acids using casein as the substrate [3]. The enzyme solutions (0.5 ml) were suitably diluted and mixed with 0.5 ml of buffer (100 mM citrate–phosphate for pH 3.5–7.0; 100 mM Tris–HCl for pH 7.0–9.0 and 100 mM glycine–NaOH for pH 9.0–11) that contained 0.5% casein, and were incubated in a water bath for 30 min at designated temperatures. The reaction was stopped by adding 0.5 ml of trichloracetic acid (20%, w/v). The mixture was allowed to settle at room temperature for 15 min, and was then centrifuged at 13,000 rpm for 15 min to remove the precipitate. The absorbance of the supernatant was measured at 280 nm. Determination of the optimum pH and apparent optimum temperatures of enzymatic reactions were performed as previously described [23]. A standard curve was generated using solutions of 0–50 mg/l tyrosine. One unit (IU) of protease activity was defined as the amount of enzyme that liberated 1 μmol of tyrosine in 1 min. Data are the average values of three or more independent experiments, unless stated otherwise.

Effect of divalent cations and inhibitors on protease activity

Divalent cations and protease inhibitors were added to the purified enzyme solution at concentrations of 5 or 10 mM [6, 18, 19]. After incubating divalent cations or inhibitors with the enzyme for 1 h at room temperature (25°C), 0.5% casein substrate was added with 50 mM Tris–HCl buffer (pH 7.5) and the mixture was incubated for 30 min at 35°C. Protease activity was detected as described before. The enzymatic activity was expressed as percentage of relative activity. Activity without inhibitors was considered to be 100%.

Results

Screening of protease-producing bacteria

One hundred and six bacterial strains were selected from sediment samples from the Aleutian margin, based on the morphological characters of their colonies on 2,216 marine agar. Forty isolates displayed a clear zone of proteolytic activity when grown on skimmed milk agar medium. Six bacteria which displayed relatively larger activity zones were selected for further study: bacterial strain D12-004 secreted protease E1; strain D3-103 secreted protease E2; strain D2-005 secreted protease E3; strain D4-001 secreted protease E4; strain D1-001 secreted protease E5; and strain D12-006 secreted protease E6.

Taxonomic identification of the protease-producing strains

BLAST analysis revealed that all six isolates were similar to bacteria of the genus *Pseudoalteromonas* (Table 1). D12-004 matched *P. issachenkonii* with a similarity of 98%, D2-005 and D4-001 closely matched *Pseudoalteromonas tetraodonis* with a similarity of 99%, and D1-001 matched *Pseudoalteromonas paragorgicola* with a similarity of 97%. D3-103 and D12-006 were affiliated with unidentified strains in the genus *Pseudoalteromonas.* The 16S rDNA sequence of strain D12-004 (*P. issachenkonii* UST041101-043) was deposited in GenBank as the code DQ178021.

pH-, temperature-, and salinity-dependent activity of the proteases

The different crude proteases that were produced by the six deep-sea bacteria formed two groups according to the optimum pH values of their protease activity (Fig. 1). The optimum pH range of the proteases E1 and E2 was pH 7–8, whereas the optimum pH of the proteases E3, E4, E5, and E6 was close to pH 9. E1 and E2 were neutral proteases, and E3, E4, E5, and E6 were alkaline proteases.

The maximal activities of proteases E1 and E2 were found at approximately 35°C, and the maximal activities for E3, E4, E5, and E6 were found at approximately 40–45°C (Fig. 2). The apparent optimal temperatures of proteases E1 and E2 were relatively low, and thus the proteases E1 and E2 could be classified as cold-adapted proteases.

Protease E1 displayed halophilic properties. It exhibited an optimum activity at a sodium chloride concentration of 2 M, which was almost double the activity in the absence of salt. In 3 M sodium chloride solution, the protease still showed a similar level of activity as in the control condition (without NaCl). However, the activity of the other five proteases decreased at elevated sodium chloride concentrations of above 1 M (Fig. 3).

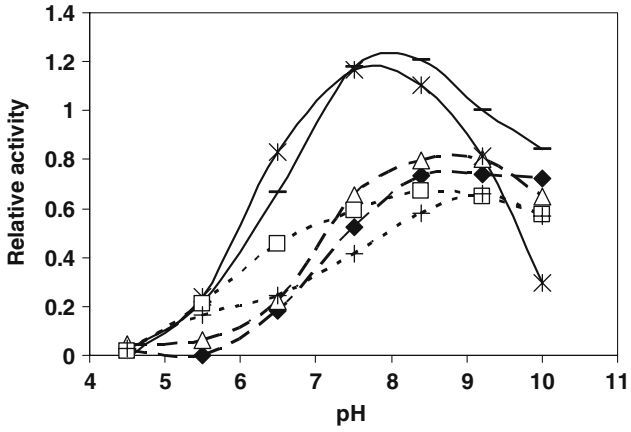


Fig. 1 pH-dependent activity of deep-sea bacterial proteases at 35°C in buffer (100 mM citrate–phosphate for pH 3.5–7.0; 100 mM Tris–HCl for pH 7.0–9.0 and 100 mM glycine–NaOH for pH 9.0–11). The proteases are marked as E1 (*asterisk*), E2 (*minus symbol*), E3 (*triangle*), E4 (*plus symbol*), E5 (*filled diamond*), and E6 (*open square*). The relative activity of the proteases is the average data

Protease purification and identification

The cold-adapted neutral halophilic protease E1 was selected for purification and further analysis. The protein purification procedures are listed in Table 2. After protein purification, the molecular weight of protease E1 was estimated to be 34 kDa by SDS-PAGE (Fig. 4). The specific activity of this protease was close to 400 IU/mg at 35°C in 50 mM of Tris–HCl buffer (pH 7.5) when casein was used as the substrate (Table 2).

Table 1 A summary of the sampling locations of the deep-sea bacteria, their taxonomic identification, and their protease codes

Strain	Closely matched species	GenBank accession number	Identities	Similarity (%)	Geographic location	Protease code
D12-004	*Pseudoalteromonas issachenkonii* UST041101-043	AF316144	954/965	98	53°17′N, 163°26′W, 3,310 m	E1
D3-103	*Pseudoalteromonas* sp. UST041101-040	AY241428	620/622	99	53°27′N, 163°22′W, 4,240 m	E2
D2-005	*Pseudoalteromonas tetraodonis* UST041101-039	AF214729	640/643	99	53°27′N, 163°22′W, 4,240 m	E3
D4-001	*Pseudoalteromonas tetraodonis* UST041101-041	AF214729	634/636	99	53°30′N, 163°27′W, 3,283 m	E4
D1-001	*Pseudoalteromonas paragorgicola* UST041101-038	AY040229	688/708	97	53°27′N, 163°22′W, 4,240 m	E5
D12-006	*Pseudoalteromonas* sp. MGP-2 UST041101-045	AF530129	665/670	99	53°17′N, 163°26′W, 3,310 m	E6

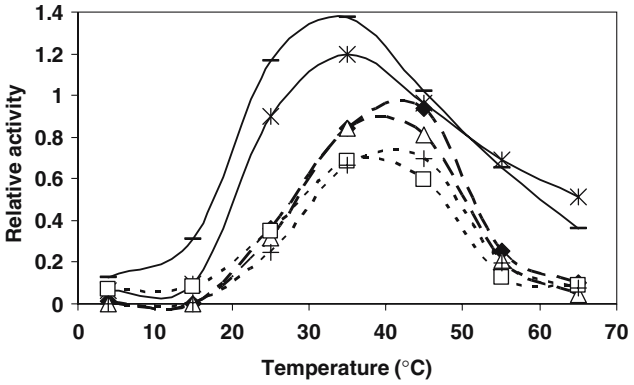


Fig. 2 Temperature-dependent activity of deep-sea bacterial proteases at pH 7 (100 mM citrate–phosphate buffer, for E1 and E2) or pH 9 (100 mM Tris–HCl buffer, for E3, E4, E5, and E6). The proteases are marked as E1 (*asterisk*), E2 (*minus symbol*), E3 (*triangle*), E4 (*plus symbol*), E5 (*filled diamond*), and E6 (*open square*). The relative activity of the proteases is the average data

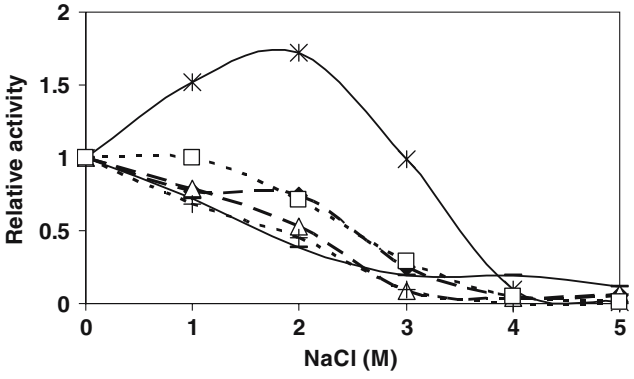


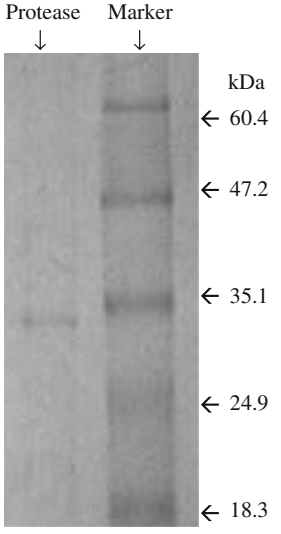
Fig. 3 NaCl-dependent activity of deep-sea bacterial proteases in buffer at 35°C and pH 7 (100 mM citrate–phosphate buffer, for E1 and E2) or pH 9 (100 mM Tris–HCl buffer, for E3, E4, E5, and E6). The proteases are marked as E1 (*asterisk*), E2 (*minus symbol*), E3 (*triangle*), E4 (*plus symbol*), E5 (*filled diamond*), and E6 (*open square*). The relative activity of the proteases is the average data

The molecular weight of protease E1 was determined to be 32,411 Da by mass spectrometric analysis (Fig. 5).

The effects of different divalent cations were tested on protease E1 activity. When added at 5 or 10 mM concentrations, only iron increased the relative activity of protease E1, when compared to the control without added cations. Zinc, copper, nickel, and manganese decreased the relative activity of protease E1. Magnesium, calcium, and barium did not significantly influence the relative activity of protease E1 (Table 3).

Testing with different protease inhibitors showed that protease E1 was a metal protease (Table 3). Only ethylenediaminetetra-acetic acid sodium salt EDTA-Na) partially inhibited the activity of the protease. At

Fig. 4 SDS-PAGE of the pure protease from *Pseudoalteromonas issachenkonii* UST041101-043. The standard protein marker (Catalog 10748-010, Invitrogen) is shown on the right side



concentrations of 5 and 10 mM EDTA, the protease retained 59 and 47%, respectively, of its activity. These results also indicated that the essential ions may hide in the protein core or tightly bound on the protein, which protect the ion removing by EDTA at short time. Phenylmethyl sulfonylfluoride (PMSF) did not inhibit the activity of protease E1 (Table 3), indicating that it was not a serine protease.

A summary of the properties of protease E1 and similar proteases from different marine bacteria is shown in Table 4. Protease E1 shows relatively more cold-adapted and halophilic enzymatic properties and different inhibition effects, when compared to the other proteases.

De novo protein sequencing

Pure protease E1 was separated by SDS-PAGE, and the protein band was determined by de novo protein sequencing. Three peptide amino acid sequences of protease E1 were reported as YNL(or I)ATTPGWDTK, WL(or I)VGQDL(or I)MK, and FL(or I)VFTR (from the N-terminal to the C-terminal). The BLAST program was used to reference these short peptide sequences against online protein sequence data. The most similar proteases were found to be those produced by *Alteromonas* sp. O-7 (BAB79615, length = 727, [14]) or *Pseudoalteromonas* sp. A28 (BAB85124, length = 731, [12]). The sequences that were most similar to YNLATTPGWDTK were metalloprotease I (BAB79615) and metal protease (BAB85124), and the sequence WLVGQDLMK was found to be similar to metalloprotease I (BAB79615) (Fig. 6).

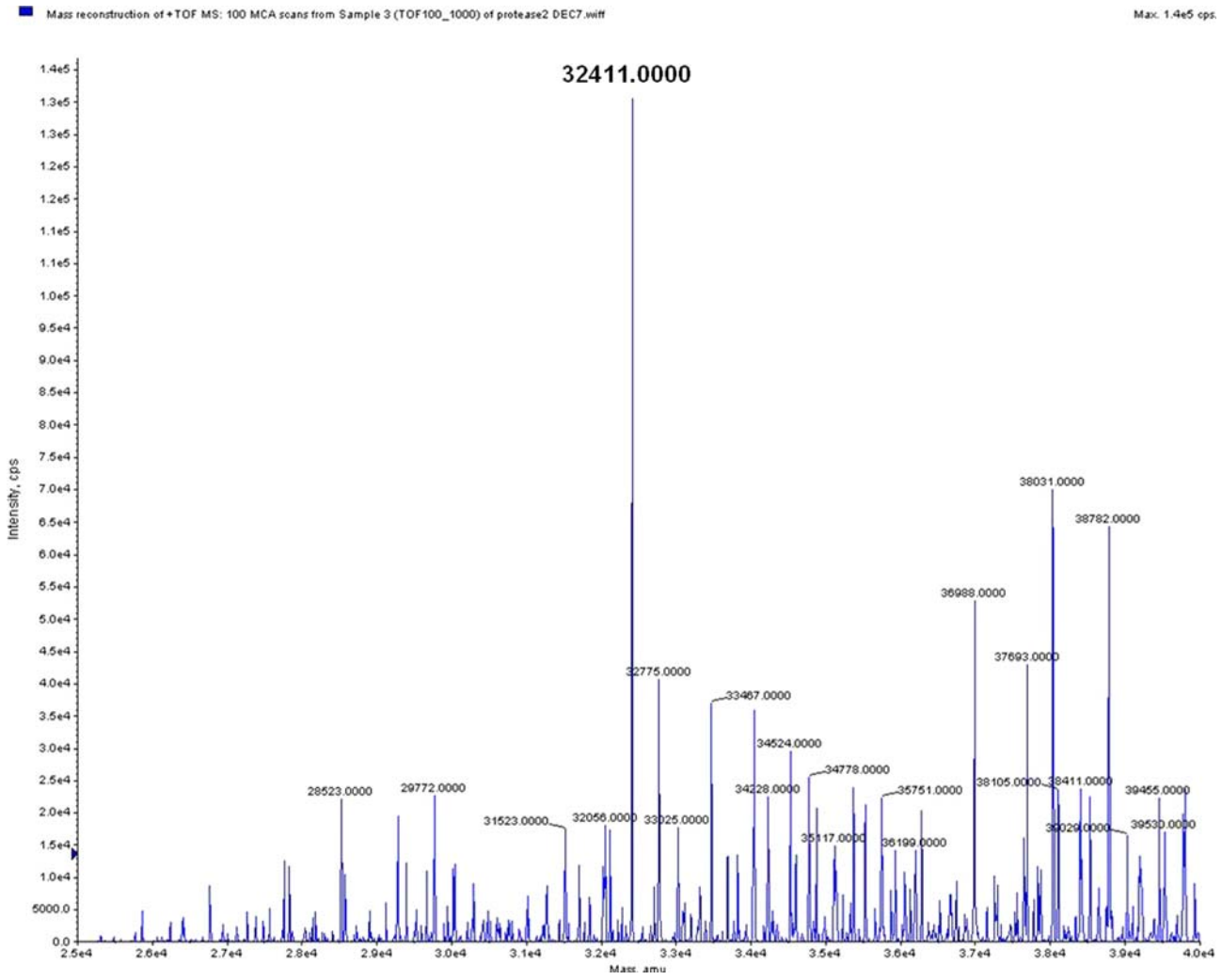


Fig. 5 ESI mass spectrometric trace of protease E1

Discussion

We have shown that the deep-sea bacterium *P. issachenkonii* UST041101-043, taken from a depth of 3,310 m in the North Pacific, produces a cold-adapted protease E1 with an apparent optimum temperature of 35°C. In the study of protein unfolding and thermostability, reports of cold-adapted enzymes can help in the explanation and design of new protein structures [20, 23]. Studies of Arctic bacteria from sea ice samples have reported the lowest apparent optimum temperature for a protease to be 20°C [7]. In that report, *Colwellia* sp. was shown to be the extracellular protease producer. A deep-sea psychrophilic bacterium *Pseudomonas* strain DY-A from a depth of 5,225 m in the East Pacific was also reported to produce a cold-adapted alkaline prote-

Table 2 Summary of the procedures for purification of the halophilic protease from *Pseudoalteromonas issachenkonii* UST041101-043

	Volume (ml)	Total activity (IU)	Relative activity (IU/ml)	Yield (%)	Specific activity (IU/mg)	Total protein (mg)
Centrifugated broth	1,500	1,923	1.3	100	ND	ND
$(NH_4)_2SO_4$ precipitate	150	1,079	7.2	56.11	ND	ND
HIC column	35	259	7.4	13.47	118	2.2
DEAE column	18	117	6.5	6.08	310	0.38
Membrane filtration	2	71	35.5	3.69	400	0.18

ND Not detected

Table 3 Effect of divalent cations and inhibitors on the protease E1 activity from *Pseudoalteromonas issachenkonii* UST041101-043

Addition	Concentration (mM)	Residual activity (%)
Blank	–	100
$CaCl_2$	5	85
	10	47
$BaCl_2$	5	90
	10	66
$CuSO_4$	5	0
	10	0
$MgCl_2$	5	93
	10	84
$FeSO_4$	5	190
	10	164
$ZnCl_2$	5	6
	10	0
$MnCl_2$	5	24
	10	0
$NiCl_2$	5	9
	10	0
EDTA	5	59
	10	47
PMSF	5	95
Tri-sodium citrate	10	94
Pepstatin	0.1	87
Okadeic acid	0.1	98
Leupeptin	0.1	99
Trypsin inhibitor	0.1	100
Antipain	0.1	100
Aprotinin	0.1	91

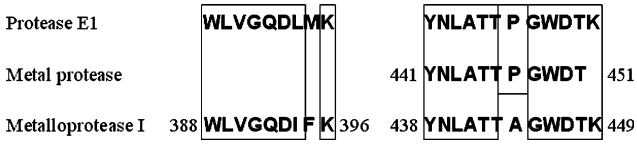


Fig. 6 Results of a National Center for Biotechnology Information BLAST search that shows the similarity between protease E1 from *Pseudoalteromonas issachenkonii* UST041101-043 and other proteases: metal protease from *Pseudoalteromonas* sp. (BAB85124) and metalloprotease I from *Alteromonas* sp. (BAB79615). The *boxes* indicate identical amino acid residues

ase, which performed at the apparent optimum temperature of 40°C [25]. Compared to these publications, the optimum temperature of the cold-adapted protease E1 was higher than the optimum temperature at which the protease produced by the polar bacterium *Colwellia* sp. but lower than the optimum temperature at which the protease was produced by the East Pacific bacterium *Pseudomonas* DY-A.

The cold-adapted protease E1 in this study displayed halophilic properties at an optimum sodium chloride concentration close to 2 M, in which condition the protease E1 performed almost double the relative activity than that without salts. Halophilic enzymes are attractive for non-aquatic or supercritical solvent medium reactions [13], as they have better thermostability and other unique properties in organic solvents [10]. Considering that organic solvents are the main diffusing reagents in the paint industry, these halophilic enzymes may have a good potential application in antifouling paints [24]. One halotolerant protease has been reported to show increased activity at elevated sodium chloride concentrations and to maintain maximum activity in 5 M sodium chloride water solution when succinyl-L-alanyl-L-alanyl-L-phenylalanine-4-nitroanilide was used as the substrate [6]. In the measurement of protease activity in halophilic proteases, most studies have used casein as the substrate [19]. However, until now no reports have been made of increasing protease activity in elevated sodium chloride concentrations when casein is used as the substrate. Thus, the finding of halophilic properties in protease E1 in this study is the first such report with casein as the substrate (Fig. 3).

Several inhibitors have been used to investigate protease properties [2, 6, 18, 19, 21]. PSMF is an efficient serine protease inhibitor, and EDTA is an efficient metalloprotease inhibitor [19, 21]. Many of the cold-adapted or halophilic proteases that have been investigated are metalloproteases or serine proteases (Table 3). For example, a halophilic serine protease was produced by *Filobacillus* sp. [6], a halophilic serine metalloprotease was produced by *Pseudoalteromonas* sp. [19] and a cold-adapted serine metalloprotease MCP-01 from *Pseudoalteromonas* sp. SM9913 [2]. The activity of protease E1 was not inhibited by PSMF, which indicates that this protease is not a serine protease. EDTA partially inhibited the activity of protease E1, which indicated that protease E1 might be a metal protease, and the essential ions of protease E1 might hide in the protein core or be tightly bound on the protein.

The molecular weight (32,411 Da) and de novo protein sequences of protease E1 are not similar to any other reported proteases according to a comparison of the similarity of protein sequence data using BLAST (NCBI, http://www.ncbi.nlm.nih.gov). The most similar metal proteases from *Alteromonas* sp. or *Pseudoalteromonas* sp. have longer sequences (727 or 731 amino acids), which are also likely to have a relatively large molecular weight. The BLAST and the molecular weight results therefore confirm that protease E1 is a novel protease because of its low molecular weight and enzymatic properties.

Table 4 Biochemical properties of proteases from different marine bacteria

Genus of protease producer	Molecular weight[a] (kDa)	Effect of inhibitor	Specific activity[b]	Optimum pH	Optimum temperature (°C)	Optimum salinity[c] (M)	Isolation location	Reference
Pseudoalteromonas issachenkonii UST041101-043	34	EDTA	400 IU/mg	7.5	35	2	53°17′N, 163°26′W, 3,310 m	This study
Alteromonas sp. O-7	56	EDTA		10	60		Sagami Bay, Japan	Miyamoto et al. [14]
Pseudomonas sp. DY-A	25	PSMF EDTA	1,423 IU/mg	10	40		8°22′N, 145°23′W 5,225 m	Zeng et al. [25]
Pseudoalteromonas sp. CP76	38	PSMF EDTA	133 IU/mg	8.5	55	1	Huelva, Spain	Sánchez-Porro et al. [19]
Filobacillus sp. RF2-5	49	PSMF	83.1 U/mg	11	60	3	Thailand	Hiraga et al. [6]
Pseudoalteromonas sp. SM9913, MCP-01	60.7	PSMF EDTA	2,890.1 U/mg	7	35		25°56′N, 125°09′E 1,855 m	Chen et al. [2]
Pseudoalteromonas sp. SM9913, MCP-02	36		536.3 U/mg	8	55		25°56′N, 125°09′E 1,855 m	Chen et al. [2]

PSMF Phenylmethyl sulfonylfuoride, *EDTA* ethylenediaminetetra-acetic acid sodium

[a] SDS-PAGE results

[b] Casein was the substrate; the enzymatic reaction condition and unit definition are indicated in the references

[c] NaCl was the salt tested

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References

1. Arahal DR, Dewhirst FE, Paster BJ, Volcani BE, Ventosa A (1996) Phylogenetic analyses of some extremely halophilic archaea isolated from Dead Sea water, determined on the basis of their 16S rRNA sequences. Appl Environ Microbiol 62:3779–3786
2. Chen XL, Zhang YZ, Gao PJ, Luan XW (2003) Two different proteases produced by a deep-sea psychrotrophic bacterial, *Pseudoaltermonas* sp. SM9913. Mar Biol 143:989–993
3. Ghorbel B, Sellami-Kamoun A, Nasri M (2003) Stability studies of protease from *Bacillus cereus* BG1. Enzyme Microb Technol 32:513–518
4. Giménez MI, Studdert CA, Sánchez JJ, Castro RED (2000) Extracellular protease of *Natrialba magadii*: purification and biochemical characterization. Extremophiles 4:181–188
5. Gupta R, Beg QK, Khan S, Chauhan B (2002) An overview on fermentation, downstream processing and properties of microbial alkaline proteases. Appl Microbiol Biotechnol 60:381–395
6. Hiraga K, Nishikata Y, Namwong S, Tanasupawat S, Takada K, Oda K (2005) Purification and characterization of serine proteinase from a halophilic bacterium, *Filobacillus* sp. RF2–5. Biosci Biotechnol Biochem 69:38–44
7. Huston AL, Krieger-Brockett BB, Demin JW (2000) Remarkably low temperature optima for extracellular enzyme activity from Arctic bacteria and sea ice. Environ Microbiol 2:383–388
8. Ivanova EP, Bakunina Y, Nedashkovskaya OI, Gorshkova NM, Alexeeva YV, Zelepuga EA, Zvaygintseva TN, Nicolau DV, Mikhailov VV (2003) Ecophysiological variabilities in ectohydrolytic enzyme activities of some *Pseudoalteromonas* species, *P. citrea, P. issachenkonii*, and *P. nigrifaciens*. Curr Microbiol 46:6–10
9. Kaye JZ, Baross JA (2004) Synchronous effects of temperature, hydrostatic pressure, and salinity on growth, phospholipid profiles, and protein patterns of four *Halomonas* species isolated from deep-sea hydrothermal-vent and sea surface environments. Appl Environ Microbiol 70:6220–6229
10. Klibanov AM (2001) Improving enzymes by using them in organic solvents. Nature 409:241–246
11. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bateriophage T4. Nature 227:680–685
12. Lee SO, Kato J, Nakashima K, Kuroda A, Ikeda T, Takiguchi N, Ohtake H (2002) Cloning and characterization of extracellular metal protease gene of the algicidal marine bacterium *Pseudoalteromonas* sp. strain A28. Biosci Biotechnol Biochem 66:1366–1369
13. Marhuenda-Egea FC, Bonete MJ (2002) Extreme halophilic enzymes in organic solvents. Curr Opin Biotechnol 13:385–389
14. Miyamoto K, Tsujibo H, Nukui E, Itoh H, Kaidzu Y, Inamori Y (2002) Isolation and characterization of the genes encoding two metalloproteases (MprI and MprII) from a marine bacte-

rium, *Alteromonas* sp. strain O-7. Biosci Biotechnol Biochem 66:416–421
15. O'Brien A, Sharp R, Russell NJ, Roller S (2004) Antarctic bacteria inhibit growth of food-borne microorganisms at low temperatures. FEMS Microbiol Ecol 48:157–167
16. Rao MB, Tanksale AM, Ghatge MS, Deshpande VV (1998) Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 62:597–635
17. Ryu K, Kim J, Dordick JS (1994) Catalytic properties and potential of an extracellular protease from an extreme halophile. Enzyme Microb Technol 16:266–275
18. Su NW, Lee MH (2001) Purification and characterization of a novel salt-tolerant protease from *Aspergillus* sp. FC-10, a soy sauce koli mold. J Ind Microbiol Biotechnol 26:254–258
19. Sánchez-Porro C, Mellado E, Bertoldo C, Antranikian G, Ventosa A (2003) Screening and characterization of the protease CP1 produced by the moderately halophilic bacterium *Pseudoalteromonas* sp. StrainCP76 Extremophiles 7:221–228
20. Turunen O, Jänis J, Fenel F, Leisola M (2004) Engineering the thermotolerance and pH optimum of family 11 xylanases by site-directed mutagenesis. Methods Enzymol 388:156–167
21. Wang SL, Chen YH, Wang CL, Yen YH, Chern MK (2005) Purification and characterization of a serine protease extracellularly produced by *Aspergillus fumigatus* in a shrimp and crab shell powder medium. Enzyme Microb Technol 36:660–665
22. Xiong H, Nyyssölä A, Jänis J, Pastinen O, Weymarn N, Leisola M, Turunen O (2004a) Characterization of the xylanase produced on submerged cultivation by *Thermomyces lanuginosus* DSM 10635. Enzyme Microb Technol 35:93–99
23. Xiong H, Fenel F, Leisola M, Turunen O (2004b) Engineering the thermostability of *Trichoderma reesei endo*-β-1,4 xylanase II by combination of disulphide bridges. Extremophiles 8:393–400
24. Yebra DM, Kiil S, Dam-Johansen K (2004) Antifouling technology—past, present and future steps towards efficient and environmentally friendly antifouling coatings. Prog Org Coat 50:75–104
25. Zeng R, Zhang R, Zhao J, Lin N (2003) Cold-active serine alkaline protease from the psychrophilic bacterium *Pseudomonas* strain DY-A: enzyme purification and characterization. Extremophiles 7:335–337