

Identification of Proteolytic Bacteria from the Arctic Chukchi Sea Expedition Cruise and Characterization of Cold-active Proteases

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Following collection of seawater samples during an Arctic Chukchi Sea expedition cruise of the Korean icebreaker Araon in 2012, a total of 15,696 bacteria were randomly isolated from Marine Broth 2216 agar plates. Of these, 2,526 (16%) showed proteolytic activity and were identified as mainly *Alteromonas* (31%), *Staphylococcus* (27%), and *Pseudoalteromonas* (14%). Among the proteolytic strains, seven were selected based on their significant ability to grow and produce a halo on skim milk plates at low temperatures (<5°C) owing to cold-active proteases. These strains were affiliated with the genus *Pseudoalteromonas* and were divided into three groups based on phylogenetic analysis of the 16S rRNA genes. Profiling cell membrane fatty acids confirmed the 16S rRNA-based differentiation and revealed the accordance between the two analyses. Seven genes for serine protease precursors were amplified from the corresponding strains, and based on sequence similarities, these genes were divided into three groups that were identical to those identified by the 16S rRNA phylogenetic analysis. Three protease genes from the representative strains of each group were composed of 2,127–2,130 bp, encoding 708–709 amino acids, and these genes yielded products with calculated molecular weights of approximately 72.3–72.8 kDa. Amino acid sequence analysis suggested that the precursors are members of the subtilase serine endo- and exo-peptidase clan and contain four domains (signal peptide, N-terminal prosequence, catalytic domain, and two pre-peptidase C-terminal domains). Upon expression in *E. coli*, each recombinant protease exhibited proteolytic activity on zymogram gels.

Keywords: Arctic, cold-active enzymes, expedition cruise, protease, *Pseudoalteromonas*

Introduction

Interest in prokaryotes from the Antarctic and Arctic is increasing especially with regard to the isolation of novel bacteria, exploration of cold-active enzymes, and development of new industrial enzymes (Nichols *et al.*, 1999; Antranikian and Egorova, 2007; Huston, 2008). Cold-adapted microorganisms isolated from low temperature habitats serve as natural bioresources for cold-active enzymes with potential industrial and commercial applications.

Proteases account for about 60% of the total industrial enzyme market (Dastager *et al.*, 2008), and the field of protease applications is diverse, including detergent preparations, food industries, meat and leather industries, and human and animal digestive aids (Rao *et al.*, 1998). For detergents, for example, cold-active proteases are added to reduce energy consumption, while in the food industry, these enzymes are used in cheese maturation and dairy production and to improve the taste of refrigerated meat products (Gerday *et al.*, 2000; Cavicchioli *et al.*, 2002; Huston, 2008). The demand for proteases, which are highly active and possess distinct biochemical features at low temperatures, in commercial enzyme markets has spurred extensive research aimed at identifying and developing novel proteases. These interests led to an exploration of the cold marine environment of the polar regions as an underexplored global niche of novel enzymes.

Materials and Methods

Sample collection and preparation

Approximately 633 marine samples, including seawater, sediments, benthic animals, and fishes, were collected at 45 different sites (stations, ST#1–ST#45; depths, surface–2,760 m) in the Chukchi Sea (73.2–82.2° N, 173° E–153° W; Fig. 1A) during the Arctic Sea expedition cruise of the Korean icebreaker Araon (August 01–September 10, 2012). Each seawater sample (1 L) from different depths was filtered through a GF/F glass microfiber filter (pore size 0.7 µm, Whatman). These samples were then stored at -20°C in 20% glycerol, which is an effective cryoprotectant for bacteria. The other samples were stored at -20°C immediately after sampling.

Isolation of cold-adapted proteolytic bacteria

At the Korea Polar Research Institute, the frozen samples were thawed at room temperature, and then a small fraction of each sample (filtered seawater, sediments, and internal organs of benthic animals and fishes) was homogenized

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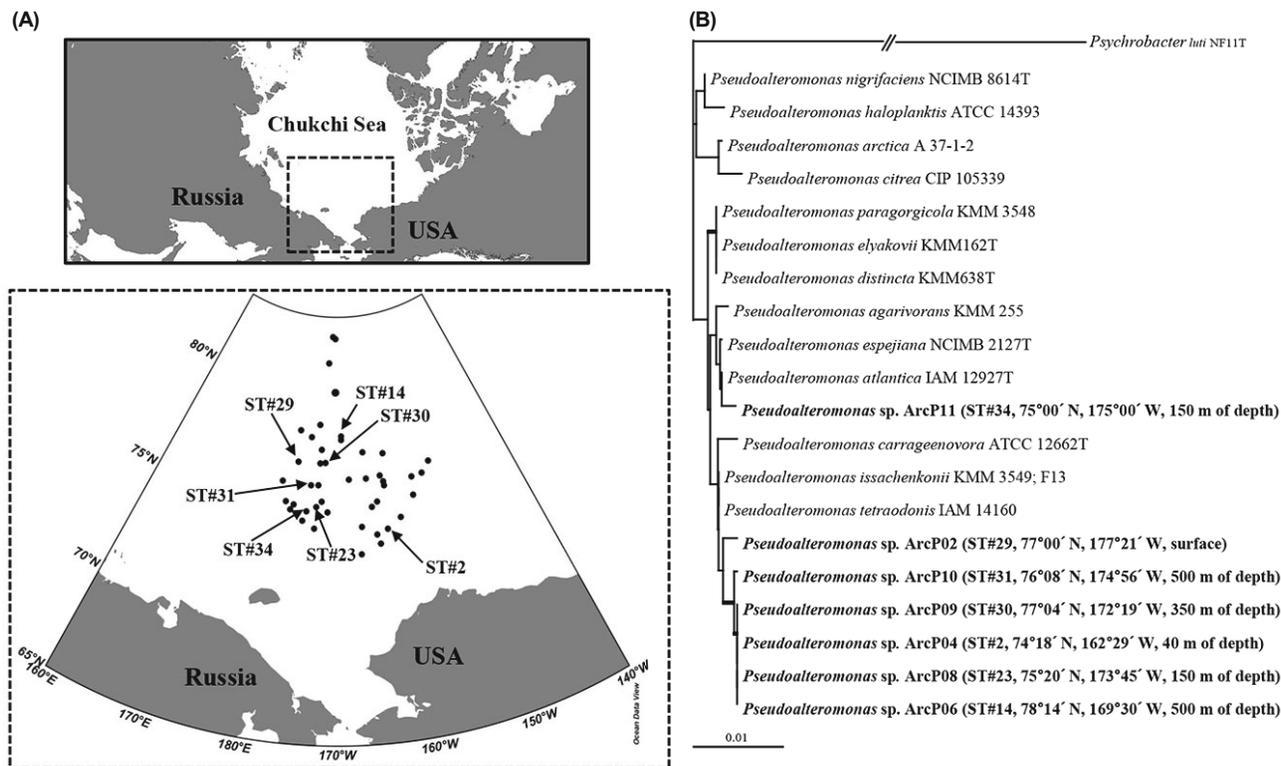


Fig. 1. The sampling sites (A) for Arctic marine *Pseudoalteromonas* strains and the phylogenetic tree generated based on 16S rRNA gene sequences (B). Detailed information (site name, GPS coordinates, and depth) of the corresponding site is shown in parentheses next to each protease-producing *Pseudoalteromonas* strain. The tree was reconstructed using the neighbor-joining method based on a distance matrix calculated by Kimura's 2-parameter evolutionary model (Kimura, 1980). The scale bar represents 0.01 substitutions per nucleotide position. Branches supported by high bootstrap values (>70%) are indicated as thick lines.

by vigorous vortexing in 20% glycerol to separate the bacteria from the sample. The supernatant (50 μ l) was spread on Difco Marine Broth 2216 (MB) agar plates and incubated at 15°C for 7 days. The bacterial colonies grown on MB agar plates were randomly picked, transferred into 700 μ l MB into individual wells of 96-well microplates, and cultured with shaking at 15°C for 7 days. After 7 days, 700 μ l of 40% glycerol was added to each well, and samples were preserved at -80°C until use. The bacterial strains were transferred from the glycerol stock onto MB agar plates containing 1% skim milk (MBS) with a 96-pin replicator and incubated at 15°C for 4 days. Proteolytic activity was scored from 0 to 4 according to the ratio of colony size to the width of the clear zone surrounding the colony as follows: '4' when the radius of clear zone was longer than diameter of colony, '3' when the radius of the clear zone was equal to the diameter of the colony, '2' when the radius of the clear zone was shorter than the diameter of the colony, '1' when a pale clear zone was observed around the colony, and '0' when no clear zone was formed. The strains with significant high activity were examined in detail for the effects of temperature on their protease production and activity following incubating on MBS at 4, 10, 15, 20, 25, 30, or 37°C for 4 days.

16S rRNA- and cellular fatty acid-based identification of bacteria

Protease-producing bacteria were chosen based on their pro-

teolytic activities, morphological characters, and isolation sites and individual bacteria were randomly selected. Partial 16S rRNA genes of these bacteria were amplified by colony-PCR with the bacterial universal primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). After sequencing using internal primers (518F, 5'-CCAGCAGCCGCGGTAATACG-3' and 800R, 5'-TACCAGGGTATCTAATCC-3'), the sequence of each strain was analyzed against 16S rRNA database using NCBI nucleotide BLAST program.

In addition to the colony PCR, conventional PCR was performed for strains that were finally selected for the ability to produce significantly high levels of cold-active protease. For this PCR, genomic DNA was extracted using LaboPass Tissue Mini kit (Cosmogenetech, Korea) and used for 16S rRNA gene amplification with the 27F and 1492R primers. The PCR product was purified using a LaboPass PCR Purification Kit (Cosmogenetech) and directly sequenced using PCR-primers (27F and 1492R) and internal primers (518F and 800R). After sequencing, the DNA sequence of each strain was assembled with a minimum of 2-fold coverage, deposited in GenBank (accession no. KF731626 to KF731632), and compared with sequences of strains available in the EzTaxon database (Chun *et al.*, 2007) to determine taxonomic affiliation. Species affiliation of a bacterial strain was determined as formation of a monophyletic group with the reference species and as 98.5% or higher similarity.

Protease-producing bacterial strains were grown on MB plates at 15°C for 3 days. Cellular fatty acids were analyzed to identify and group bacteria. Fatty acid methyl esters were prepared through five steps (harvesting, saponification, methylation, extraction, and base wash) as described by Sasser (1990) and separated by gas chromatography (Agilent Technologies 6850). The fatty acid methyl ester composition was compared to compositions recorded in a stored database with the Sherlock pattern recognition software (Microbial ID, USA).

Protease enzyme assay

Bacteria were cultivated in 50 ml MBS medium for 3 days at 15°C, and the culture was centrifuged (10,000 × *g* for 30 min at 4°C). An aliquot (0.2 ml) of the supernatant (crude enzyme solution) was added to 0.9 ml of 0.65% azocasein solution in 50 mM Tris-HCl (pH 8.0, standard buffer). After incubation for 1 h at 30°C, the reaction was terminated by addition of 0.9 ml of 110 mM trichloroacetic acid solution, and the precipitate was removed by centrifugation at 12,000 × *g* for 3 min. As a reference, the enzyme solution was added to a solution of standard buffer containing azocasein after the addition of trichloroacetic acid, and the reaction was centrifuged. An aliquot (0.5 ml) of the supernatant, which contained amino acids that were released from azocasein, was mixed with 0.25 ml Folin-Ciocalteu's phenol reagent (Sigma) and 1.25 ml of 0.5 M Na₂CO₃. After incubation at 25°C for 30 min, the solution was cleared by a brief centrifugation. The absorbance was measured at 660 nm in a cuvette with a 1-cm light path, and the amount of released amino acids was calculated by comparison to a standard curve of L-tyrosine. One unit of protease activity was defined as liberation of 1 nmol amino acids/min/mg of protein at 30°C. The concentration of protein was determined with BSA as a standard.

Protease classification

The main protease in a crude enzyme solution was classified according to the extent of enzymatic inhibition by a class-specific chemical as described below. An aliquot (200 µl) of the enzyme solution was added to 810 µl of the standard buffer supplemented with various protease inhibitors (1 mM phenylmethanesulfonyl fluoride [PMSF]; 1 mM 1,10-phenanthroline; 1 mM *N*-ethylmaleimide; 0.3 mM pepstatin A) and incubated for 1 h on ice. Following incubation, 90 µl of a 6.5% azocasein solution was added, and the samples were further incubated for 1 h at 30°C. The residual proteolytic activity was calculated relative to the activity of an untreated control reaction (100%), as described in the protease enzyme assay section above.

Effects of temperature and pH on protease activity and stability

The optimal temperature for proteolytic activity was determined following addition of the enzyme solution and azocasein to the standard buffer that had been pre-treated at a given temperature in the range of 0–60°C. After a 1-h incubation, the relative activity was measured. The thermal stability was analyzed via reaction at 30°C followed by measure-

ment of the residual activity of each enzyme solution that had been pre-incubated at various temperatures (0–60°C) for 1 h. Subtilisin Carlsberg (3 µg) was used as control enzyme at different temperatures.

The optimum pH for proteolytic activity was measured in 1.1 ml of reaction mixture containing 0.65% azocasein and the enzyme solution after incubating at 30°C for 1 h. The pH was adjusted by using the following buffers: 50 mM sodium acetate (pH 4.0–6.0) and 50 mM Tris-HCl (pH 7.0–10.0). For the determination of pH stability, each of the enzyme solutions (100 µl) was pre-incubated in 900 µl of different buffers with various pH ranges on ice for 1 h, which were 50 mM sodium acetate (pH 2.0–6.0), 50 mM Tris-HCl (pH 7.0–10.0). Two hundred microliter of treated enzymes were added to 0.9 ml of standard buffer containing 0.65% azocasein and incubated at 30°C for 1 h. Subtilisin Carlsberg (3 µg) was used as control enzyme at 55°C.

Partial cloning and sequencing of protease genes

Genomic DNA was prepared from bacteria grown in MB using a G-spin Genomic DNA Extraction Kit (iNtRON Biotechnology, Korea). One degenerate primer set (ASH-F and ASS-R) was designed to amplify a region between highly conserved active-site histidine and serine residues in serine proteases: ASH-F (5'-CAY GGN ACN CAY GTN GCN GG-3') and ASS-R (5'-NGG NGC CAY NGA NGT NCC-3'). PCR reactions were performed in a 20-µl reaction mixture containing 1× PCR reaction buffer, 200 µM dNTPs, 0.2 µM of each primer, 200 ng of genomic DNA, and 1 unit of Taq DNA polymerase (Bioneer Inc., Korea). The PCR cycling conditions were as follows: an initial denaturing step at 95°C for 5 min, 30 cycles of amplification (95°C for 1 min, 59.5°C for 1 min, and 72°C for 1 min), and a final extension at 72°C for 10 min. The PCR product was purified using a LaboPass Gel Extraction Kit (CosmoGenetech) and ligated into the pCR2.1-TOPO vector (Invitrogen). The recombinant vector was transferred into *E. coli* DH5α cells, and then the insert DNA was sequenced with M13 Forward (5'-GTA AAA CGA CGG CCA G-3') and M13 Reverse (5'-CAG GAA ACA GCT ATG AC-3') primers.

Complete cloning and heterologous expression of serine protease genes

Based on the GenBank DNA sequences from Arctic marine bacteria that showed the highest identities with those of the partial serine protease genes, two serine protease-specific primer sets were designed as follows: SP1-F (5'-ATG ACT TCT AAT AAT TCA TTC-3') and SP1-R (5'-TTA TGG CTG GTA TTG ACC GCT-3') and SP2-F (5'-ATG ACA ACA AGT AAA TCA TTT-3') and SP2-R (5'-TTA TGG CTG GTA TTG GCC ACT-3'). Using these primer sets and extracted genomic DNA, serine proteases were PCR-amplified with Prime STAR HS Taq Polymerase (TaKaRa Bio Inc., Japan) by denaturation at 95°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 3 min for a total of 30 cycles. The PCR product was purified, adenylated with Taq polymerase, cloned into expression vector pEXP5-CT/TOPO (Invitrogen) and sequenced. The DNA sequence of each protease was deposited in GenBank (accession no.

Table 1. Fatty acid profiles (%) of Arctic marine *Pseudoalteromonas* strains

Fatty acid	ArcP02	ArcP04	ArcP06	ArcP08	ArcP09	ArcP10	ArcP11
Straight-chain							
C _{12:0}	2.0	2.3	2.4	2.3	2.3	2.3	2.5
C _{16:0}	19.1	21.5	19.7	21.8	20.5	20.8	18.3
C _{17:0}	4.5	2.5	3.8	2.3	3.1	2.8	3.6
C _{18:0}	1.8	1.7	2.4	2.1	1.9	1.9	1.5
Unsaturated							
C _{17:1} ω8c	9.2	4.6	7.4	3.8	5.6	5.2	5.7
Hydroxy							
C _{10:0} 3-OH	tr	tr	tr	tr	1.1	tr	1.4
C _{12:0} 3-OH	5.4	6.0	6.1	6.1	6.1	6.4	5.8
Sum in Feature 3*	32.3	33.7	29.7	32.4	31.0	31.3	31.5
Sum in Feature 7*	1.3	1.2	tr	1.0	1.2	1.1	tr
Sum in Feature 8*	12.9	17.1	15.2	18.2	17.1	17.3	20.9

*Sum in feature represents fatty acids that could not be separated by GLC with the MIDI system: Sum in Feature 3 comprises C_{16:1} ω7c or C_{16:1} ω6c; Sum in Feature 7, C_{19:1} ω7c or C_{19:1} ω6c; Sum in Feature 8, C_{18:1} ω7c. tr, trace (<1.0%). Fatty acids amounting to <1.0% of the total acids in all strains were omitted.

KF731619 to KF731625). After transformation of *E. coli* BL21 Star (DE3) (Invitrogen), one single colony was grown in 50 ml LB supplemented with 100 µg/ml ampicillin at 37°C until an OD₆₀₀=1.0 was reached. The culture was maintained at 15°C for 1 h, and then heterologous expression was induced with 1.0 mM IPTG at 15°C for 3 days. After centrifugation (10,000 × g, 30 min, 4°C), the cell-free supernatant was concentrated through a 10-kDa cut-off Vivaspin (Satorius Inc., Germany). Finally, each 500 µl of the concentrate was used for SDS-PAGE and zymogram analyses.

Results

Selection of proteolytic bacteria from Arctic Chukchi Sea

Following incubation of 633 different samples at 15°C for 7 days, bacterial colonies appeared on MB agar plates mainly from samples that originated from seawater. Some of the bacterial colonies exhibited different colony shapes or colors, although most strains appeared similar to one another. Among the bacteria from the seawater samples, 15,696 were randomly selected and preserved as 20% glycerol stocks in 96-well microplates. Upon incubation on MBS plates at 15°C for 4 days, 2,526 bacterial strains (16%) produced a clear zone around the colonies. To gain information for the taxonomical distribution of culturable protease-producing bacteria from the Chukchi Sea, 111 bacteria were randomly selected from the 2,526 strains and examined for their morphological characters and isolation sites. The 16S rRNA genes were found to be taxonomically associated with three major taxa: genus *Alteromonas* (31%), *Staphylococcus* (27%), and *Pseudoalteromonas* (14%). The minor taxa were *Leeuwenhoekella* (7%), *Bacillus* (5%), *Sulfitobacter* (5%), *Psychrobacter* (4%), *Croceibacter* (2%), *Acinetobacter* (2%), *Pseudomonas* (1%), *Halomonas* (1%), and *Dokdonia* (1%).

The 2,526 strains were considered as cold-adapted proteolytic bacteria and scored according to the size of the clear zone on the plates. Although all of the colonies produced a clear zone (score 1–4) at 15°C, only seven strains produced a large zone and were scored as a '4'. When the seven strains were re-examined under the same experimental conditions

in temperatures ranging from 4–37°C, all of the strains produced clear zones at temperatures as low as 5°C but were unable to produce these clear zones at temperatures above 30°C. This characteristic is indicative of cold-adapted hosts producing cold-active proteases.

Identification and grouping of proteolytic bacteria

Incidentally, the seven strains (designated ArcP02, ArcP04, ArcP06, ArcP08, ArcP09, ArcP10, and ArcP11) were from seawater filtrates of different sites (station ST#2-#34) and depths (sea surface–500 m under the surface) (Figs. 1A and 1B). According to the sequence similarity and phylogenetic analysis of the 16S rRNA gene sequences, these bacteria were all identified as *Pseudoalteromonas* sp. strains. In the phylogenetic tree, these *Pseudoalteromonas* strains formed three clades. *Pseudoalteromonas* sp. ArcP11 had 99.9% similarity to *P. atlantica* and was monophyletic with that strain, while the other six strains that had 99.9% to 100% similarities with *P. tetraodonis* were not monophyletic with any known strains (Fig. 1B).

To determine whether the seven strains are chemotaxonomically separable into discrete groups similar to those determined by 16S rRNA gene sequences, the cell membrane fatty acid profiles were analyzed following growth on MB agar plates for 3 days at 25°C (Table 1). The predominant fatty acids of all strains were Sum in Feature 3 (29.7–33.7%); however, several significant differences in fatty acid composition were identified between the bacteria. For example, a secondary major fatty acid in ArcP11 was Sum in Feature 8 (20.9%) and was C_{16:0} (19.1–21.8%) in ArcP02, ArcP04, ArcP06, ArcP08, ArcP09, and ArcP10. The proportion in C_{17:1} ω8c and C_{12:0} 3-OH (9.2% and 5.4%, respectively) in ArcP02 was significantly different than those in ArcP04, ArcP06, ArcP08, ArcP09, ArcP10, and ArcP11 (3.8–7.4% and 5.8–6.4%, respectively). This analysis showed that these strains are divided into three groups according to the fatty acid composition of the cell membrane as follows: ArcP02, ArcP04–ArcP06–ArcP08–ArcP09–ArcP10, and ArcP11. These results are in concordance with the grouping based on the phylogenetic relationships of the 16S rRNA genes.

Table 2. Effects of protease inhibitors on protease activity from Arctic marine *Pseudoalteromonas* strains

Reagent	Inhibitor type	Final concentration	Residual activity (%)		
			ArcP02	ArcP08	ArcP11
No inhibitor	-	-	100	100	100
PMSF	Serine	1.0 mM	75.2±5.2	60.2±2.8	68.6±3.9
1,10-Phenanthroline	Metallo	1.0 mM	72.7±4.9	78.8±9.0	97.0±8.5
N-Ethylmaleimide	Cysteine	1.0 mM	113.7±3.5	101.8±6.9	80.4±4.9
Pepstatin A	Aspartic acid	0.3 mM	95.0±7.9	85.9±6.1	95.9±4.0

Protease classification

The above mentioned plate assay revealed that seven *Pseudoalteromonas* strains (ArcP02-ArcP11) produced a clear zone at temperatures of 4–25°C, potentially due to the presence of cold-active proteases. Although proteases do not easily comply with a general system of enzymatic nomenclature due to their huge variations in both action and structure, these enzymes can be classified into four groups based on the chemical nature of the catalytic site and/or the functional group present at the active site: serine protease, aspartic protease, cysteine protease, and metalloprotease. Thus, proteases from the three cold-adapted strains (ArcP02, ArcP08, and ArcP11), which were selected as a representative of each 16S rRNA-based group, were classified according to the extent of enzymatic inhibition by class-specific chemicals (Table 2). A greater than 20% decrease in protease activity following inhibitor treatment compared to untreated controls (100%) constituted a negative effect. The activities of crude protease solutions from ArcP02, ArcP08, and ArcP11 decreased to 75.2%, 60.2%, and 68.6%, respectively, by the serine protease inhibitor PMSF. Interestingly, the protease

solutions obtained from ArcP02 and ArcP08 were partially inhibited by 72.7% and 78.8%, respectively, by the metalloprotease inhibitor 1, 10-phenanthroline, while that of ArcP11 was not (97.0% residual activity). Rao and co-workers (Rao *et al.*, 1998) reported that microbial serine proteases are numerous and widespread among diverse taxa, suggesting their vital importance to microorganisms. Accordingly, these data indicate that ArcP02 and ArcP08 may additionally produce metalloprotease, except for serine protease.

Effects of temperature and pH on protease activity and stability

To determine the optimal temperature and thermostability of the serine proteases, supernatants were obtained from cell cultures grown at 15°C, and their proteolytic activities were measured at temperatures ranging from 0–60°C. As the reaction temperature increased from 0°C to 50°C, the protease activities increased with a maximal activity at 40–50°C. Interestingly, the relative activities of ArcP02, ArcP08, and ArcP11 at 0°C were 48%, 41%, and 32%, respectively, in comparison to 100% at the optimal temperature of 40°C

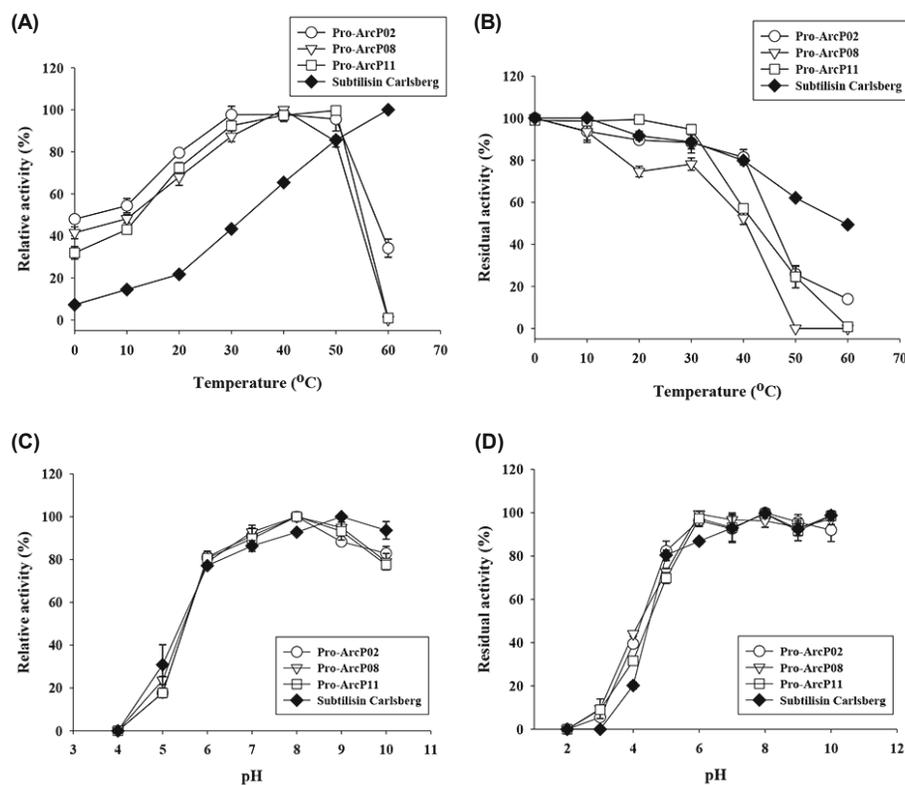


Fig. 2. Determination of optimal temperature (A) and pH (C), and stability over a broad range of temperatures (B) and pHs (D) of extracellular proteases from Arctic marine *Pseudoalteromonas* strains. Mesophilic serine protease subtilisin Carlsberg (3 µg) was tested under the same conditions as those of *Pseudoalteromonas* strains.

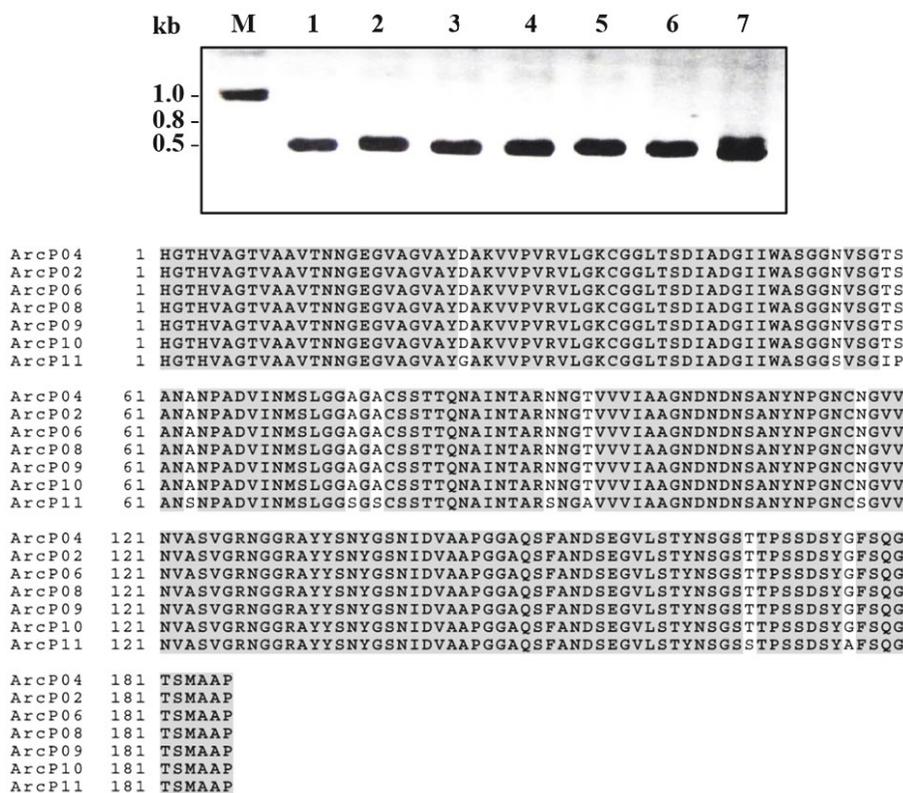


Fig. 3. PCR amplification (upper) of partial protease genes and sequence alignment (lower) of the PCR products from Arctic marine *Pseudoalteromonas* strains. Lanes: M, DNA size marker; 1, ArcP02; 2, ArcP04; 3, ArcP06; 4, ArcP08; 5, ArcP09; 6, ArcP10; 7, ArcP11. Abbreviations in lower: ArcP02–ArcP11, serine protease from *Pseudoalteromonas* sp. ArcP02–ArcP11.

(Fig. 2A). Pre-incubation of the proteases at 0–60°C resulted in significantly different effects; the enzyme activities slowly decreased with increasing temperatures until temperatures reached 30°C but rapidly decreased at temperatures higher than 40°C (Fig. 2B). In contrast, the commercially available mesophilic serine protease subtilisin Carlsberg exhibited relatively lower activities at temperatures ranging from 0–20°C with a maximal activity at 60°C. This protease maintained its activity up to approximately 80% at 40°C. These experimental data demonstrate that the three proteases from Arctic marine *Pseudoalteromonas* strains possess at least one feature of cold-adapted enzymes in contrast to mesophilic proteases. Specifically, these proteases exhibit higher activity at low temperatures such as less than 10°C.

The effect of pH was determined by pre-incubating enzymes in buffer solutions of various pH values. All three enzymes exhibited its higher activity at pH 7.0–9.0 with maximum activity at pH 8.0 (Fig. 2C). The enzymes remained stable in a broad range of pH 5.0–10.0, but lost its activity at pHs lower than 5.0 (Fig. 2D), suggesting the alkaline nature of the enzyme. In accordance with the three enzymes, alkaline subtilisin Carlsberg was stable between pH 5.0 and 10.0 with maximum activity at pH 8.0, and below pH 5.0 rapidly lost the activity.

PCR amplification and sequence analysis of protease genes

The protease gene fragments were amplified via PCR from genomic DNA of the seven strains using the degenerate primer set (ASH-F and ASS-R), which was designed for amplification of a region between highly conserved active-site

residues in bacterial serine proteases. Approximately 558-bp DNA fragments were amplified from all strains and then sequenced and comparatively analyzed by amino acid alignment. Amino acid sequences from all strains were completely identical to each other, except the ArcP11 sequence differed from the other strains (Fig. 3).

Two specific primer sets (SP1-F/SP1-R and SP2-F/SP2-R) were designed based on the nucleotide sequences of serine proteases from marine *Pseudoalteromonas* spp. with the highest identity with the N-terminal regions of the partial PCR products from the representative strains (ArcP02, ArcP08, and ArcP11) from each 16S rRNA analysis-based group. The full sequences of serine proteases from ArcP02 and ArcP08 were amplified with SP1-F and SP1-R primers, while the protease from ArcP11 was amplified with SP2-F and SP2-R primers. Each PCR product, which was composed of 2,127 bp (ArcP11)–2,130 bp (ArcP02 and ArcP08), encoded 708–709 amino acids and had a calculated molecular weight of approximately 72.3–72.8 kDa.

The amino acid sequences deduced from the nucleotide sequences were submitted to NCBI protein-protein BLAST and CD-Search programs. The protein sequences showed 89–99% identity with serine protease precursors composed of 708–711 amino acids from different *Pseudoalteromonas* sp. strains that originated from cold seawater. For example, the identities of the serine protease precursors from *Pseudoalteromonas* sp. ArcP02, ArcP08, and ArcP11 showed 99%, 99%, and 89% similarities, respectively, with the serine protease precursor from *Pseudoalteromonas* sp. SM9913. CD-Search identified a catalytic domain that contained three conserved amino acid residues (for ArcP02, Asp 188-His

ArcP02	1	MTSNN SFKKCAVALTITITLFAASSSMANPAQA IAP SMAETS AKLQ N GGGFETQFI IKYKN
ArcP08	1	MTSNN SFKKCAVALTIS TLFAASSSMANPAQA IAP SMAETS AKLQ NGGGFETQFI IKYKN
MCP-03	1	MTSNN SFKKCAVALTIS TLFAASSSMANPAQA IAP SMAETS AKLQ NGGGFETQFI IKYKN
ArcP11	1	MTTSK SFKKCAIALTIS TVFAASASVA --- QVST SMAET TAKLQ S SGGFETQFI IKYKN
ArcP02	61	-NNDMMSTSTADVSPAMNKKKQ S FVKNFTSKKGGKVKAKYVRAMALNNHHVMRADKKLNA
ArcP08	61	-NNDMMSTSTADVSPAMNKKKQ S FVKNFTSKKGGKVKAKYVRAMALNNHHVMRADKKLNT
MCP-03	61	-NNDMMSTSTADVSPAMNKKKQ S FVKNFTSKKGGKVKAKYVRAMALNNHHVMRADKKLNA
ArcP11	58	SANDMLSIS S ADAS P ANMKKRA Q R F VKNFASKGGKVKAKYIRAMALNNHHVMRADKKLSA
ArcP02	120	EAAQ Q FMQEMVNSGNVEYIEVDQMLK P FATPNDRYGDQ W HYYEQAGGLNLP T AWDTATG
ArcP08	120	EAAQ Q FMQEMVNSGNVEYIEVDQMLK P FATPNDRYGDQ W HYYEQAGGLNLP T AWDTATG
MCP-03	120	EAAQ Q FMQEMVNSGNVEYIEVDQMLK P FATPNDRYGDQ W HYYEQAGGLNLP T AWDTATG
ArcP11	118	AEAQ E FMQEMVNSGNVEYIEVDQMLK P FATPNDRYGDQ W HYYEQAGGLNLP T AWDTATG
ArcP02	180	SGVVAVLD T GYRPHADLNANILPGYDMISNLSVANDGNGRDN D ARDPGDAVAAGECGNN
ArcP08	180	SGVVAVLD T GYRPHADLNANILPGYDMISNLSVANDGNGRDN D ARDPGDAVAAGECGNN
MCP-03	180	SGVVAVLD T GYRPHADLNANILPGYDMISNLSVANDGNGRDN D ARDPGDAVAAGECGNN
ArcP11	178	SGVVAVLD T GYRPHVDLNANILPGYDMISNLSVANDGGGRD S DARDPGDAVAAGECGNN
ArcP02	240	GAQGS S WHGTHVAGTVAAVTNNGEVAGVAYDAKVV P RVRLGKCGGLTSDIADGI I WASG
ArcP08	240	GAQGS S WHGTHVAGTVAAVTNNGEVAGVAYDAKVV P RVRLGKCGGLTSDIADGI I WASG
MCP-03	240	GAQGS S WHGTHVAGTVAAVTNNGEVAGVAYDAKVV P RVRLGKCGGLTSDIADGI I WASG
ArcP11	238	GAQGS S WHGTHVAGTVAAVTNNGEVAGVAYDAKVV P RVRLGKCGGLTSDIADGI I WASG
ArcP02	300	GNVSGT S ANANPADVINMSLGGAGAC S STTQNAINTARNNGT V VVIAAGNDNDNSANYNP
ArcP08	300	GNVSGT S ANANPADVINMSLGGAGAC S STTQNAINTARNNGT V VVIAAGNDNDNSANYNP
MCP-03	300	GNVSGT S ANANPADVINMSLGGAGAC S STTQNAINTARNNGT V VVIAAGNDNDNSANYNP
ArcP11	298	GSVSG I PANSNPADVINMSLGG S CSSTTQNAINTARSNGAVV I AAGNDNDNSANYNP
ArcP02	360	GNCNGVVN V ASVGRNNGRAYYSNYGS N IDVAAPGGAQ S FANDSEGL V LSTYNSG S TTPSSD
ArcP08	360	GNCNGVVN V ASVGRNNGRAYYSNYGS N IDVAAPGGAQ S FANDSEGL V LSTYNSG S TTPSSD
MCP-03	360	GNCNGVVN V ASVGRNNGRAYYSNYGS N IDVAAPGGAQ S FANDSEGL V LSTYNSG S TTPSSD
ArcP11	358	GNC S GVVN V ASVGRNNGRAYYSNYGS N IDVAAPGGAQ S FANDSEGL V LSTYNSG S TTPSSD
ArcP02	420	SYGFS Q GT S MAAPHVAGVAALIKQAKPNAT P DEIESILKTT R TRPFSATCT S CGT G I V DA A
ArcP08	420	SYGFS Q GT S MAAPHVAGVAALIKQAKPNAT P DEIESILKTT R TRPFSATCT S CGT G I V DA A
MCP-03	420	SYGFS Q GT S MAAPHVAGVAALIKQAKPNAT P DEIESILKTT R TRPFSATCT S CGT G I V DA A
ArcP11	418	SYAF S QGT S MAAPHVAGVAALIKQAKPNAT P DEIESILK S T R TRPFSATCT S CGT G I V DA A
ArcP02	480	AAVAAASGG-TPPTTGDNELVDGEVKTGLSGTAS A QDFYTM T VP S GATN V TF T MSGGT G D
ArcP08	480	AAVAAASGG-TPPTTGDNELVDGEVKTGLSGAAS A QDFYTM T VP S GATN V TF T MSAGT G D
MCP-03	480	AAVAAASGG-TPPTTGDNELVDGEVKTGLSGAAS A QDFYTM T VP S GATN V TF T MSGGT G D
ArcP11	478	AAVAAASGGTTPPTGG S ELTDGEAKTGLSGAAS S QAYY T M T VP S GATN V TF T MSGG S GD
ArcP02	539	ADLYVRAGSKP S STTYDCR P YKGGNS E ECSD I DNPTAGTYH V MLRGYS A YS G VS I VGNIT G
ArcP08	539	ADLYVRAGSKP S STTYDCR P YKGGNS E ECSD I DNPTAGTYH V MLRGYS A YS G VS I VGNIT G
MCP-03	539	ADLYVRAGSKP S STTYDCR P YKGGNS E ECSD I DNPTAGTYH V MLRGYS A YS G VS I VGNIT G
ArcP11	538	ADLYVRAGSQ P TT S SYDCR P YKGGNS E ECSD I DNPTAGTYH V MLRGYS A YS G VS I VGNIT G
ArcP02	599	GSTGGGSGT P QAGGGT V SDITANA G QWKHY T LDV P AGMAS F T V TT S GGT G DADL F V K F G S
ArcP08	599	GSTGGGSGT P QAGGGT V SDITANA G QWKHY T LDV P AGMAS F T V TT S GGT G DADL F V K F G S
MCP-03	599	GSTGGGSGT P QAGGGT V SDITANA G QWKHY T LDV P AGMAN F T V TT S GGT G DADL F V K F G S
ArcP11	598	GSTGGGSGT P KAGGGT V SDVTA T IGNWEHY T LDV P AGM S T F T V TT S GGT G DADL F V K F G S
ArcP02	659	QPTSS Y DCR P YKNGNA E TCT F SNP Q AGT W HLS V NAY Q T F S G L T L S GG Y Q P
ArcP08	659	QPTSS Y DCR P YKNGNA E TCT F SNP Q AGT W HLS V NAY Q T F S G L T L S GG Y Q P
MCP-03	659	QPTSS Y DCR P YKNGNA E TCT F SNP Q AGT W HLS V NAY Q T F S G L T L S GG Y Q P
ArcP11	658	QPTAS S YDCR P YKNGNA E TCT F SNP Q AGT W HLS V NAY R T F S G L T L S GG Y Q P

247-Ser 428), which constitutes a catalytic triad similar to that found in trypsin-like proteases, and assigned the catalytic domain as a member of peptidases S8 family or subtilases serine endo- and exo-peptidase clan. To obtain more information about the presence of functional domains, the protein sequences were aligned with one functionally characterized homologue (cold-active protease), MCP-03 from *Pseudoalteromonas* sp. SM9913 (Yan *et al.*, 2009). Based on the results of the sequence alignments, the following four domains were predicted: a signal peptide for enzyme secretion, an N-terminal prosequence for correct folding, a catalytic domain, and two bacterial pre-peptidase C-terminal domains for enzyme thermostability (Fig. 4).

Cloning and heterologous expression of serine protease genes

The serine protease gene was amplified from each strain: *pro-arcP02* from ArcP02, *pro-arcP08* from ArcP08, and *pro-arcP11* from ArcP11. The PCR products were then cloned into the expression vector pEXP5-CT/TOPO, which was transferred into *E. coli* BL21 Star (DE3) cells. Following heterologous expression and centrifugation, the supernatant that contained each recombinant protease was analyzed for size and activity using SDS-PAGE and zymogram analyses. SDS-PAGE analysis did not reveal overexpression of any of the

Fig. 4. Sequence alignment of proteases from Arctic marine *Pseudoalteromonas* strains and homologous MCP-03. The predicted signal peptide is underlined with one line. The first amino acid residue (P) of the mature protease is indicated by an arrow. The catalytic triad (D, H, and S) is indicated by asterisks. Two bacterial pre-peptidase C-terminal domains are in dotted-line boxes. Abbreviations: MCP-03, cold-adapted halophilic serine protease MCP-03 (GenBank accession No. DQ422814) from *Pseudoalteromonas* sp. SM9913; ArcP02, serine protease from *Pseudoalteromonas* sp. ArcP02; ArcP08, serine protease from *Pseudoalteromonas* sp. ArcP08; ArcP11, serine protease from *Pseudoalteromonas* sp. ArcP11.

proteases in *E. coli* (Fig. 5A); however, zymogram analysis clearly revealed clear zone(s) after incubation at 4°C, and the bands corresponded to molecules of 50–100 kDa (Fig. 5B).

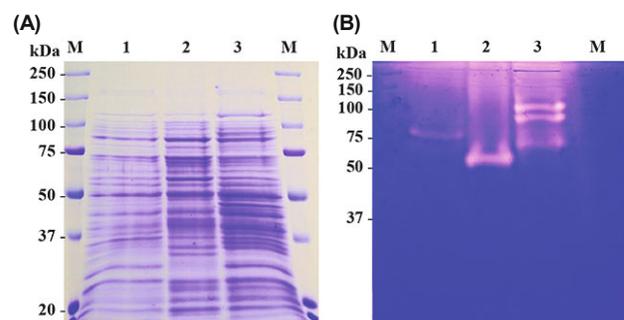


Fig. 5. SDS-PAGE (A) and zymogram (B) analyses of recombinant proteases of Arctic marine *Pseudoalteromonas* strains. SDS-PAGE analysis of 500 μ l of each concentrated culture supernatant was performed on 10% polyacrylamide slab gels using Tris-glycine buffer system. For zymogram analysis, the supernatant was separated on 10% SDS-PAGE gels containing 1.0% skim milk, and the gel was rinsed in 100 ml renaturing buffer (27 g Triton X-100 in 1 L distilled water) and 100 ml developing buffer (1.21 g Tris base, 6.3 g Tris-HCl, 11.7 g NaCl, 0.74 g CaCl₂, 0.02% Brij 35 in 1 L distilled water) for 30 min. The rinsed gel was incubated in 100 ml developing buffer at 4°C for 4 h and stained with Coomassie Blue R-250. Lanes: M, protein size marker; 1, ArcP02; 2, ArcP08; 3, ArcP11.

Discussion

The Gram-negative heterotrophic and aerobic *Pseudoalteromonas* is present globally in marine environments and constitutes 0.5–6.0% of the total bacterioplankton. These species are divided into two groups, pigmented and non-pigmented strains. Since the non-pigmented strains have highly similar 16S rRNA gene sequences, this similarity precludes differentiation below species level using 16S rRNA data (Wietz *et al.*, 2010; Vynne *et al.*, 2011); however, in present study, the seven non-pigmented *Pseudoalteromonas* strains, which were selected due to their high cold-activity from the 2,526 proteolytic isolates from different Arctic Chukchi Sea sites, were divided into three groups by 16S rRNA-based phylogenetic analysis: *Pseudoalteromonas* sp. ArcP02; *Pseudoalteromonas* sp. ArcP04, ArcP06, ArcP08, ArcP09, and ArcP10; and *Pseudoalteromonas atlantica* ArcP11. In addition, the fatty acid cell membrane profiling, which served as a chemotaxonomical approach, confirmed the 16S rRNA-based differentiation.

The protease production of marine bacteria including *Pseudoalteromonas* is a trait involved in nutrient utilization and turnover of marine microorganisms on a global scale. Indeed, many psychrophilic and psychrotolerant strains of marine *Pseudoalteromonas* produce a variety of extracellular cold-active proteases (Vázquez *et al.*, 2008). This characteristic may help bacteria and surrounding microbes to use various proteins as nutrients. Characterizing distinct features, such as structure-function relationships and substrates, could improve understanding of the ecological roles of marine microbes, including *Pseudoalteromonas*, particularly in cold marine environments. It was stated that cold-adapted microorganisms can either synthesize cold-active enzymes or produce high levels of enzymes to support nutrient degradation and uptake (Feller and Gerday, 1997). The synthesis of catabolic enzymes specifically adapted to low temperatures is believed to be a mechanism of adaptation to the cold, which is used by psychrophiles at the enzyme level (Collins *et al.*, 2008).

When considering the prevalence of proteases in marine environments and the physiological variation derived from adaptation to local specific niches of *Pseudoalteromonas* strains, we speculated that each of these proteolytic bacteria produce a different type of protease that represents the group (in this case, a 16S rRNA-based monophyletic group). Indeed, the cold-active serine proteases from the seven strains were divided into three groups based on their amino acid sequences: Pro-ArcP02; Pro-ArcP04, -ArcP06, -ArcP08, -ArcP09, and -ArcP10; Pro-ArcP11. This finding was consistent with our hypothesis. In conclusion, this work demonstrated that multiple strains of one *Pseudoalteromonas* species can be divided by 16S rRNA-based phylogenetic analysis, and each strain may possess different protease enzymes due to intra-species variations and/or specific adaptation to different environments according to ecophysiology. However, these data were not sufficient to generalize the presence of a specific protease in a 16S rRNA-based phylogenetic group. Additional investigation with marine *Pseudoalteromonas* strains from various locations is necessary to examine the possibility of a phylogenetic group-specific protease.

As proteases remain the best way to remove protein stains and all commercial detergent proteases are from bacteria, bacterial cold-active serine proteases are considered as new additive enzymes in laundry detergents and also expected to open up new markets in the developing world. In the present study, three serine proteases from *Pseudoalteromonas* strains (ArcP02, ArcP08, and ArcP11) showed higher relative activities at low temperatures (32–48% at 0°C in comparison to 100% at the optimal temperature) than that of subtilisin Carlsberg that is produced at commercial level as the trademark Alcalase by Novozymes. Polarzyme, a new low-temperature detergent enzyme from Novozymes, showed a relative activity of 35% at 10°C in comparison to 100% activity at optimal 50°C (data not shown). To be used in the detergents, besides higher cold-activity at low temperatures, other unique characters are additionally needed, such as higher activity at the pHs of detergent-containing wash water. Therefore, cold-active alkaline serine proteases may be successful at low washing temperatures. ArcP02, ArcP08, and ArcP11 displayed higher activity and stability at alkaline pH 8.0–10.0, which was comparable with those of subtilisin Carlsberg. Taking into account of the overall results, it can be concluded that these serine-type alkaline proteases may be added to commercial detergent formula, as a new psychrophilic detergent enzyme, either alone or mixed with other mesophilic enzymes.

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