J. Microbiol. Biotechnol. (2018), 28(3), 448–453
https://doi.org/10.4014/jmb.1711.11006

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Purification, Characterization, and Cloning of a Cold-Adapted Protease from Antarctic *Janthinobacterium lividum*

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Received: November 3, 2017
Revised: November 30, 2017
Accepted: December 2, 2017

First published online
December 8, 2017

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pISSN 1017-7825, eISSN 1738-8872

In this study, a 107 kDa protease from psychrophilic *Janthinobacterium lividum* PAMC 26541 was purified by anion-exchange chromatography. The specific activity of the purified protease was 264 U/mg, and the overall yield was 12.5%. The *J. lividum* PAMC 25641 protease showed optimal activity at pH 7.0–7.5 and 40°C. Protease activity was inhibited by PMSF, but not by DTT. On the basis of the N-terminal sequence of the purified protease, the gene encoding the cold-adapted protease from *J. lividum* PAMC 25641 was cloned into the pET-28a(+) vector and heterologously expressed in *Escherichia coli* BL21(DE3) as an intracellular soluble protein.

Keywords: Cold-adapted protease, *Janthinobacterium lividum*, purification, expression

Among the various organisms living in extreme environments, psychrophilic (cold-adapted) microorganisms are the most abundant in the world [1]. Psychrophiles have been isolated from cold environments such as the polar regions, terrestrial soils, glaciers, and oceans. They have attracted considerable attention because they are known to produce enzymes that have high activity at moderate and low temperatures, which would contribute to lower energy requirements and costs related to heat treatment [2].

Proteases are hydrolases that cleave the peptide bonds in proteins and peptides. They are used in a wide variety of industrial applications, such as detergent production, leather processing, food manufacture, pharmaceutical processes, and environmental bioremediation, accounting for appropriately 60% of the worldwide enzyme market [3]. In comparison with their mesophilic and thermophilic counterparts, cold-adapted proteases possess high specific activity at low and moderate temperatures and increased structural flexibility [4]. Consequently, cold-adapted proteases have potential for use in various industrial applications [5].

The identification of novel proteases with new catalytic properties is of great importance for enzyme research and potential industrial applications. However, only some cold-adapted proteases have been identified from psychrophilic and psychrotolerant microbes such as *Stenotrophomonas maltophilia*, *Penicillium chrysogenum*, and *Azospirillum* sp. [6–9]. Further information on the characterization and genetic structure of various cold-adapted proteases is required. In the present study, we describe the purification and biochemical characterization of a cold-adapted protease from a novel Antarctic strain, *Janthinobacterium lividum* PAMC 25641. Furthermore, the gene encoding the cold-adapted protease was successfully cloned and heterologously expressed in *Escherichia coli*.

J. lividum PAMC 25641 was obtained from the Polar BioCenter (Korea Polar Research Institute, Korea), and its protease activity was confirmed using *N*-succinyl-AAPF-p-nitroanilide (AAPF) (Sigma-Aldrich, USA) as a substrate. *J. lividum* PAMC 25641 was grown in nutrient broth (Becton, Dickinson and Company, USA) at 15°C for 72 h with constant shaking at 150 rpm. *Escherichia coli* TOP10 (Invitrogen, USA) and *E. coli* BL21(DE3) (Invitrogen) were used as host strains for cloning and expression, respectively, and were grown in Lysogeny Bertani (LB) broth at 37°C.

The plasmid pET-28a(+) (Invitrogen) containing a kanamycin resistance gene was used as a cloning and expression vector.

For enzyme purification, the clear culture supernatant (3 days after inoculation) was salted out with ammonium sulfate to 80% saturation. The precipitated proteins were separated by centrifugation at 15,000 ×*g* for 30 min at 4°C. The pellet was dissolved in 20 mM Tris-HCl buffer (pH 8.5) and thoroughly dialyzed with at least two changes of the same buffer. The dialyzed supernatant was applied to a DEAE-Sepharose column (1.5 × 5 cm) equilibrated with 100 ml of 20 mM Tris-HCl buffer (pH 8.5). Proteins were eluted with 20 mM Tris-HCl buffer (pH 8.5) using a linear gradient of NaCl solution (0–0.5 M) at a flow rate of 30 ml/h, with 10 ml of the fraction volume. Then, the fractions were assayed for enzyme activity.

Protein concentration was determined by the Bradford assay [10]. The absorbance was measured at 595 nm, and bovine serum albumin was used as a standard for calibration. Protease activity was determined using AAPF as the substrate. The reaction mixture included 200 µl of 50 mM sodium phosphate buffer (pH 7.2), 100 µl of enzyme solution, 10 µl of 10 mM AAPF, and 690 µl of double-distilled water. The reaction mixture was incubated for 10 min at 25°C and then inactivated for 20 min at 80°C. The absorbance of the sample was measured at 410 nm using an ELISA reader (Molecular Devices, USA). A unit of protease production was calculated using the extinction coefficient (ε) of 8,800 $l \cdot mol^{-1} \cdot cm^{-1}$ and the following formula [11]:

$$\text{Unit } (\mu mol/min)/l = \frac{(\text{net } OD_{410}) \times (\text{volume of reaction mixture, L}) \times 10^6}{\varepsilon \times (\text{reaction time, min}) \times (\text{volume of enzyme solution, L})}$$

The purification fold was obtained by dividing the specific activity of each step by the initial specific activity, and the yield (%) was calculated by dividing the total activity of each step by the initial total activity and multiplying by 100.

The effect of pH on the purified protease activity was determined by incubating the reaction mixture at different pH values. The pH was adjusted with the following buffers (10 mM); sodium acetate-acetic acid (pH 4.0–5.5), potassium phosphate (pH 6.0–8.0), Tris-HCl (pH 8.0–9.0), and sodium carbonate-sodium hydrogen carbonate (pH 9.5–10.5). The reaction mixtures were incubated for 10 min at 25°C, and the activity of the enzyme was measured.

The optimum temperature for enzyme activity was determined by assaying the enzyme at different temperatures ranging from 5°C to 60°C in 10 mM sodium phosphate buffer (pH 7.2) for 10 min.

To investigate the effects of metal ions and inhibitors on protease activity, the purified protease was incubated in reaction mixtures with various metal ions (10 mM K^+, Na^+, Mg^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, and Zn^{2+}) and inhibitors (1 mM Tween-20, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 3% ethanol). The activity of the enzyme without any additives was considered as 100%.

All experiments were carried out in triplicate, and the values are presented as the mean ± standard deviation.

The purified protease was detected by SDS-PAGE as described by Laemmli [12] and transferred to a polyvinylidene difluoride membrane with 10 mM CAPS buffer. The membrane was stained with Coomassie brilliant blue and destained until the bands were visible. The stained band was excised and N-terminal amino acid sequencing was performed using a Procise protein sequencing system (Applied Biosystems, USA). The first 10 residues of the N-terminal amino acid sequence of the purified protease was analyzed using partial genomic DNA sequence databases of *J. lividum* PAMC 25641 obtained by a MiSeq sequencing system (Illumina, USA).

The open reading frame of the protease from *J. lividum* PAMC 25641 was analyzed using the NCBI ORF Finder tool (http://www.ncbi.him.nih.gov/gorf/gorf/gorf.html) and the InterPro tool (https://www.ebi.ac.uk/interpro/). The signal peptide sequence was predicted using the SignaIP 4.0 Server online program (http://www.cbs.dtu.dk/services/SignalP/).

For amplifying the open reading frame of the protease gene, which was identified by N-terminal amino acid sequencing and genomic DNA sequence analysis, a forward primer (5′-TAA GCA CAT ATG AAA CTA CGT CCC GTT-3′, with the NdeI restriction site underlined) and reverse primer (5′-TAA GCA AAG CTT TTA TTC AGC CAG CAC CG-3′, with the HindIII restriction site underlined) were designed. The purified PCR products and pET-28a(+) vector were digested by NdeI and HindIII, and ligated to obtain the recombinant plasmid pET28a-pro. The confirmed pET28a-pro plasmid was transformed into *E. coli* BL21(DE3) for heterologous expression.

E. coli BL21(DE3) carrying the recombinant plasmid pET28a-pro was cultured overnight at 37°C in LB broth containing 30 µg/ml of kanamycin. An aliquot of the overnight culture (1% (v/v)) was inoculated into 100 ml of fresh LB broth containing 30 µg/ml of kanamycin and grown at 37°C until the OD_{600} reached 0.4–0.6. Then, 0.1 mM

isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce the heterologous expression of the protease overnight at 20°C. The lysate pellet and supernatant of the cell lysate were subjected to SDS-PAGE for analysis of the soluble recombinant protein. The molecular mass of the protease was estimated using 8% and 12% polyacrylamide gels with defined protein markers (BioPrince, Korea).

J. lividum PAMC 25641 demonstrated the highest protease activity among the Antarctic microorganisms obtained from the Polar BioCenter (Korea Polar Research Institute), as previously described [13]. Therefore, the protease from *J. lividum* PAMC 25641 was selected for purification and characterization in this study.

Proteins were precipitated with 80% saturation of ammonium sulfate and purified by anion-exchange chromatography. The final amount of the purified protease was 3 mg, and the overall yield was 12.5% with a specific activity of 264 U/mg. SDS-PAGE analysis showed a single band associated with the purified protease, and its molecular mass was about 110 kDa (Fig. 1).

The pH and temperature activity profiles of the purified protease are illustrated in Fig. 2. The optimal pH of the protease was pH 7.0–7.5 with a specific activity of 264 U/mg, indicating that the purified protease belongs to a neutral class of protease [3]. Moreover, the protease remained active within a broad range of pH values (pH 6.0–9.0) with a specific activity of between 214 and 264 U/mg. On the other hand, the protease activity declined sharply in the pH ranges of 4.0–5.5 and 9.5–10.5, retaining less than 50% of its maximal activity (Fig. 2A). Therefore, the purified protease with high activity in the neutral to alkaline pH range may be suitable for biotechnological applications, since neutral and alkaline proteases are used widely in many industrial applications such as detergents and food processing [14].

As shown in Fig. 2B, a maximum activity of 322 U/mg was observed at 40°C, which was similar to the activity of cold-adapted proteases from *Alkaliphilus transvaalensis* and *Exiguobacterium* sp. SKPB5 [15, 16]. Moreover, the protease maintained its catalytic activity between 10°C and 35°C with a specific activity between 247 and 283 U/mg. Even

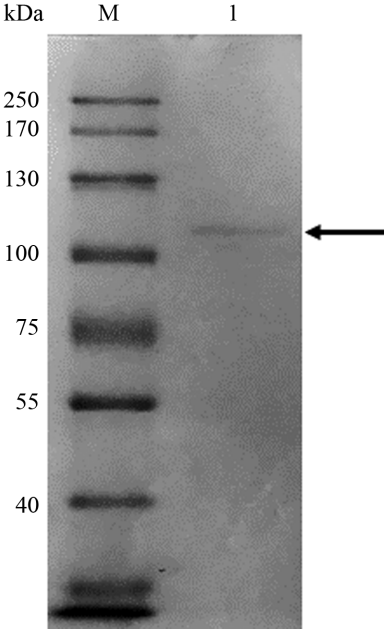

Fig. 1. SDS-PAGE analysis of the purified protease from *Janthinobacterium lividum* PAMC 25641.
M, standard protein marker; 1, purified protease.

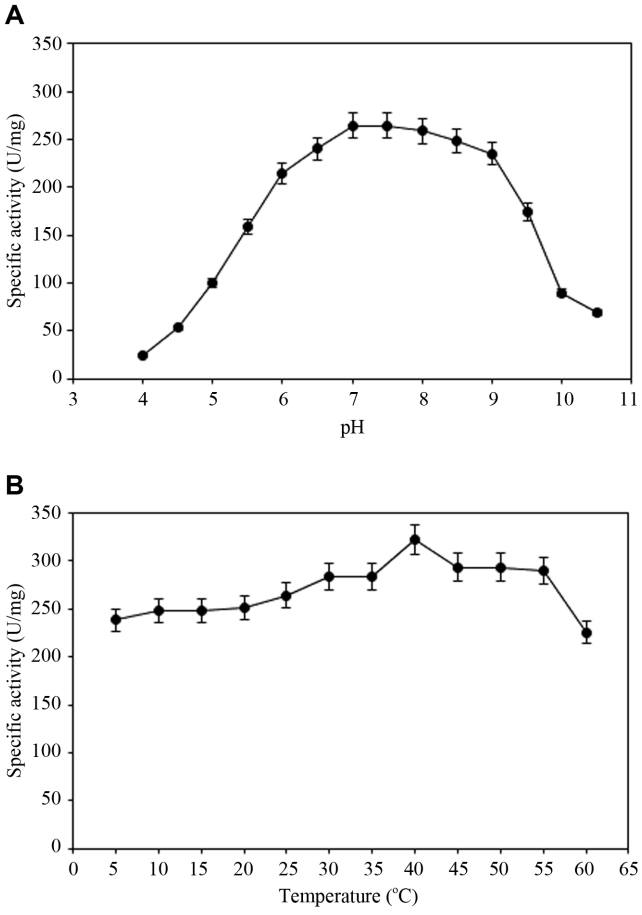

Fig. 2. Effects of pH and temperature on the activity of the purified protease from *Janthinobacterium lividum* PAMC 26541.
(**A**) Enzyme activity was measured at various pH values (4.0–10.5) for 10 min at 25°C. (**B**) Enzyme activity was determined at different temperatures ranging from 5°C to 60°C for 10 min at pH 7.2.

within a temperature range of 45–55°C, the enzyme still effectively retained its activity above 290 U/mg (Fig. 2B). In the case of the thermostable alkaline protease from *Bacillus* sp., the optimum temperature was 70°C and it retained only 50% of its maximal activity at 30°C [17]. These results indicated that the *J. lividum* PAMC 25641 protease belongs to the cold-adapted enzyme class. In general, the activity of typical cold-adapted proteases would shift from maximum to minimum at low temperatures as well as at higher temperature because of thermolability. For example, the cold-adapted protease from *Planococcus* sp. has maximal activity at 35°C and would be rapidly inactivated at temperatures above 35°C [6]. However, the *J. lividum* PAMC 25641 protease demonstrated high catalytic activity within a broad range of temperatures (25–55°C).

The effects of different metal ions and chemical compounds on the activity of the purified protease are shown in Table 1. Protease activity was inhibited in the presence of 10 mM Ca^{2+}, Co^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, and Zn^{2+} ions with a residual activity of 18%, 11%, 7%, 6%, 4%, and 2%, respectively. However, the enzyme activity was stimulated by K^{+} and Na^{+} ions with a residual activity of 118% and 115%, respectively.

Among all the chemical reagents tested, PMSF was found to inhibit the enzyme activity, indicating that this purified protease belongs to the serine protease family [18]. With the addition of 1 mM PMSF, the enzyme retained 45% of activity. PMSF may inhibit enzyme activity by sulfonating the serine residue in the active site of the protease [19]. However, the proteolytic activity of this cold-adapted protease was not completely inhibited by PMSF. In a previous study, a thermostable alkaline protease retained only 5% activity in the presence of 1 mM PMSF [17]. The enzyme in our study was partially inhibited by the sulfhydryl reagent β-mercaptoethanol. On the other hand, DTT did not inhibit the enzyme activity, indicating that the protease activity is not affected by disulfide bonding [6]. In addition, the enzyme retained more than 90% of its initial activity in the presence of Tween-20, ethanol, and EDTA. The stability of the enzyme in the presence of EDTA indicated that metallic ions are not required for enzyme activity [20]. This is a beneficial property in the detergent industry because EDTA is one of the ingredients in detergents [21].

Table 1. Effect of various metal ions and chemical reagents on the activity of the purified protease from *Janthinobacterium lividum* PAMC 26541.

Reagent	Concentration	Relative enzyme activity (%)
(Control)	None	100 ± 4
K_2SO_4	10 mM	118 ± 7
Na_2SO_4	10 mM	115 ± 3
$MgSO_4$	10 mM	87 ± 3
$CaCl_2$	10 mM	18 ± 6
$CoCl_2$	10 mM	11 ± 2
$MnSO_4$	10 mM	7 ± 10
$FeSO_4$	10 mM	6 ± 8
$CuSO_4$	10 mM	4 ± 6
$ZnSO_4$	10 mM	2 ± 5
Tween-20	1 mM	99 ± 3
EDTA	1 mM	91 ± 5
DTT	1 mM	90 ± 4
β-Mercaptoethanol	1 mM	85 ± 7
PMSF	1 mM	45 ± 5
Ethanol	3%	96 ± 3

The sequence of the first 10 amino acid residues of the purified protease was determined to be TSYTPSFLGL by N-terminal sequencing. The amino acid sequence was identical to the gene encoding a putative protease from the partial genomic DNA sequence databases of *J. lividum* PAMC 25641 (data not shown), and its open reading frame was identified.

An approximately 3 kb fragment of the protease gene was amplified using the genomic DNA of *J. lividum* as the template. The PCR product was inserted into the pET-28a(+) vector and transformed into *E. coli* TOP10 for cloning and sequencing. The nucleotide sequence of the protease was deposited in the GenBank database under the accession number KX688889. Sequence analysis revealed an open reading frame of 3,165 nucleotides encoding 1,054 amino acid residues. By using the ExPASy server (http://www.expasy.org/), the molecular mass and theoretical pI of the recombinant protease were predicted to be 106.6 kDa and 6.39, respectively.

A putative signal peptide containing 20 amino acid residues was detected by SignaIP 4.0 (http://www.cbs.dtu.dk/services/SignalP/), suggesting that the protease from *J. lividum* PAMC 25641 might be an extracellular enzyme. The amino acid sequence of the *J. lividum* PAMC 25641 protease exhibited 67% identity to the serine protease (subtilase family) from *Janthinobacterium* sp. CG23-2 (CUI07110.1), 52% identity to the S8 proteases from *Massilia* sp. LC238 (WP_036216271.1), *Pseudoduganella violaceinigra* (WP_035372814.1), and *Janthinobacterium agaricidamnosum* (WP_038500374.1), and 51% identity to the S8 protease from *J. lividum*

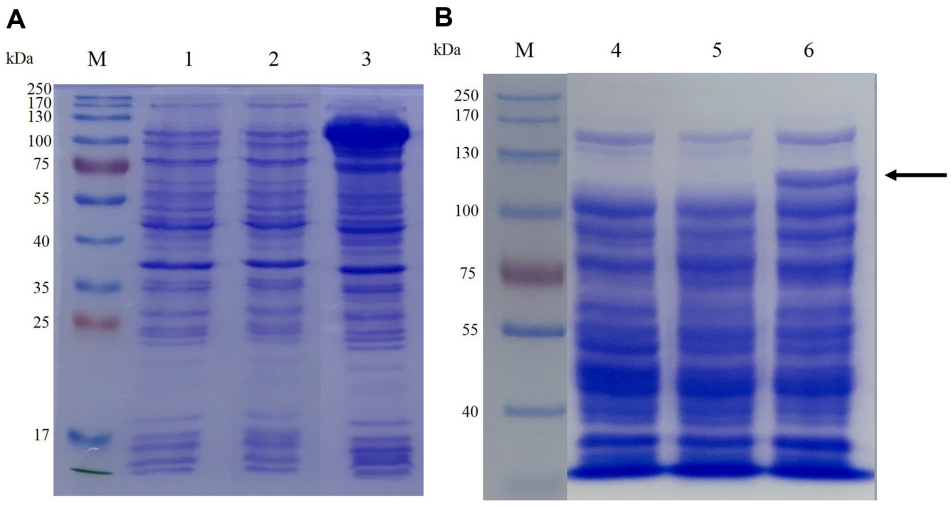

Fig. 3. Expression of the recombinant protease from *Janthinobacterium lividum* PAMC 26541 in *E. coli*.
(**A**) SDS-PAGE analysis (12% polyacrylamide gel) of recombinant *E. coli* cells. M, Standard protein marker; 1, cell pellet containing pET-28a(+); 2, cell pellet containing pET28a-pro before induction; 3, cell pellet containing pET28a-pro after induction. (**B**) SDS-PAGE analysis (8% polyacrylamide gel) of cell lysate. M, Standard protein marker; 4, supernatant of cell lysate (soluble proteins) containing pET-28a(+); 5, supernatant of cell lysate containing pET28a-pro before induction; 6, supernatant of cell lysate containing pET28a-pro after induction.

(WP_034782163.1). Nucleotide sequence alignment with other proteases revealed that the primary structure of the protease contains six highly conserved regions (regions I–VI) and three catalytic triads (Asp-166, His-255, and Ser-612).

The recombinant plasmid pET28a-pro was transformed into *E. coli* BL21(DE3). The recombinant protease was successfully expressed by adding 0.1 mM IPTG to the culture medium (Fig. 3A). After the disruption of cells by sonication, SDS-PAGE (8% polyacrylamide gel) analysis showed that the recombinant protein was approximately 110 kDa in the soluble fraction and insoluble fraction of the cell lysate (Fig. 3B). The protease in the soluble fraction of the cell lysate had a specific activity of 17.22 U/mg; however, there was no protease activity in the supernatant of the cell culture. Moreover, protease activity was not detected in the *E. coli* BL21(pET-28a(+)) and non-induced *E. coli* BL21(pET28a-pro) transformants. These results indicated that the recombinant protease was heterologously expressed in the soluble form.

In conclusion, we purified a novel cold-adapted protease from *J. lividium* and carried out the characterization of the protease. Furthermore, with the N-terminal sequencing and partial genomic sequence data, the gene encoding the protease was cloned and expressed in *E. coli*. These results will be useful for the structural characterization and industrial application of cold-adapted proteins.

Acknowledgments

This study was financially supported by a National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (No. NRF-2015R1A2A2A01004733), and by the Golden Seed Project (213008-05-2-SB910), Ministry of Agriculture, Ministry of Oceans and Fisheries.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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