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Effect of Temperature on the Yield, Activity, and Stability of a Cold-Active Protease from *Pseudoalteromonas arctica* PAMC 21717

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Abstract

Pseudoalteromonas arctica *PAMC 21717 is a psychrophilic microorganism, which was originally isolated from Antarctica. This psychrophile produces the cold-active, extracellular, serine-type protease W-Pro21717. In the present study, we examined the effect of temperature on the cellular growth* of P. arctica*, its protease yield, and the stability and activity of the enzyme. The optimal temperatures for the highest yield and protease activity were determined to be 15℃ and 40℃, respectively. W-Pro21717 exhibited relatively high activity even at low temperatures close to 0℃. However, it showed lower thermal stability than a mesophilic protease, which is characteristic of a psychrophilic enzyme. The half-life of W-Pro21717 was estimated to be 0.36 h at 40℃. These findings supplement our understanding of cold-active proteases and may have commercial applications.*

Keywords: Cold-active protease, *Pseudoalteromonas arctica* PAMC 21717, W-Pro21717, Thermal stability, Psychrophile

1. Introduction

There has been increasing interest in the commercial use and applications of cold-active enzymes in biotechnology (GEORLETTE & al. [1]). Psychrophilic enzymes from cold-adapted microorganisms have higher catalytic efficiencies than their mesophilic counterparts, especially at low temperatures. Cold-active proteases in particular are commercially useful, especially as laundry detergent enzymes, owing to their high activity during washing processes that utilize cold tap water. Until date, several protease-producing, psychrotrophic microorganisms have been identified (KASANA [2]). Typically, the optimal growth conditions for these microorganisms are determined so as to ensure a high yield for industrial applications (WANG & al. [3], PRANAW & al. [4]). It is also necessary to determine the optimal temperature conditions for the activity of a cold-active protease of interest, in order to make its use commercially viable.

In our previous study, we identified *Pseudoalteromonas arctica* PAMC 21717 from the Polar and Alpine Microbial Collection (PAMC) as a microbe of interest because it secretes a high level of a psychrophilic, alkaline serine protease (KIM & al. [5]). Subsequently, we named the protease W-Pro21717 and enhanced enzyme production by medium optimization and using the fed-batch culture mode. This resulted in a 15-fold increment in the production of enzyme from microbial cultures (HAN & al. [6]). Temperature is one of the most influential factors that

determine the productivity of a psychrophilic enzyme (KIM & al. [7], HAN & al. [8]). Therefore, this study attempted to investigate the effect of temperature on the growth of *P. arctica* PAMC 21717 and on the productivity, activity, and stability of W-Pro21717.

2. Materials and methods

2.1. Microbial strain and media composition

P. arctica PAMC 21717 was isolated from a soil sample collected from the Barton Peninsula (S 62°13′, W 58°47′) on King George Island, Antarctica. A seed culture was prepared by inoculating cells in 50 mL of Zobell's medium (MOSKOT & al. [9]) and incubating it for 24 h at 15°C. This seed culture (10%) was used to inoculate modified Marine Broth medium to facilitate cell growth and production of the cold-active protease. The modified Marine Broth medium was composed of skim milk (10 g/L), $Fe(C_6H_5O_7)$ (ferric citrate; 0.1 g/L), NaCl (24.8 g/L), Na_2SO_4 (4.4 g/L), KCl (1.8 g/L), $NaHCO_3$ (0.16 g/L), KBr (0.08 g/L), and tryptone (0.6 g/L) (ZOBELL [10]).

2.2. Production of W-Pro21717

Batch cultures were prepared using 50 mL of modified Marine Broth medium in a 250 mL Erlenmeyer flask. The 50 mL batches were incubated at various culture temperatures of 10, 15, 20, and 25°C and shaken at 150 rpm. Growth of *P. arctica* PAMC 21717 was monitored by measuring the optical density of the cultures at 600 nm (OD_{600}), using a spectrophotometer (S-3100; Scinco, Seoul, Korea).

2.3. Thermal characterization of W-Pro21717

The optimum temperature for W-Pro21717 activity was determined by measuring the enzyme activities at temperatures ranging from 0°C to 60°C. W-Pro21717 activity was compared with that of a commercially available mesophilic protease from *Bacillus licheniformis* (P-5380; Sigma-Aldrich Inc., St. Louis, MO, USA). To investigate the thermal stability of W-Pro21717 and the mesophilic protease, samples were incubated at various temperatures ranging from 0°C to 60°C for 1 h, and then activity was determined at 40°C. Additionally, the proteases were incubated at 40°C for periods ranging from 0 to 3 h, and then the retained activities were measured at their optimal temperatures. The half-life of the enzyme was calculated as the incubation time required for protease activity to reduce to 50% of the initial activity.

2.4. Enzyme assay

W-Pro21717 activity in the broth was measured, using N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPF-pNA, Sigma-Aldrich Inc., St. Louis, MO, USA) as a substrate. The cultured broth was centrifuged (12,000 × *g* for 5 min) and the supernatant was used as the enzyme (W-Pro21717) solution. At least three different volumes of the W-Pro21717 solution (10–100 μL) were diluted to 1 mL(final volume) using the standard buffer containing the substrate (10 mM of sodium phosphate buffer at pH 7.5, 1 mM of substrate). These reaction mixtures were incubated for 10 min. The amount of *p*-nitroaniline produced as a result of enzyme activity was calculated by measuring the OD_{410}, using a molar extinction coefficient of 8,800 $M^{-1}cm^{-1}$. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 μmol substrate to *p*-nitroaniline per minute.

3. Results and discussion

3.1. Effect of culture temperature on W-Pro21717 yield

P. arctica PAMC 21717 was cultivated at different temperatures of 10, 15, 20, and 25°C for 72 h and cell densities of the culture and activities of W-Pro21717 were measured. Cultures showed maximal cell density of OD_{600}=4.83 at 15°C (Fig. 1). At the same temperature, W-Pro21717 had maximal specific activity of 0.19 U/mg (Fig. 1). A comparison of the W-Pro21717 activity with the previously reported protease activities would be burdensome because of the differences in the units and substrates used (GUPTA & al. [11]). The specific

unit of W-Pro21717 is lower than that of the serine protease reported as a novel, extracellular hydrolase from *Kluyveromyces marxianus* IFO 0288 (FOUKIS & al. [12]).

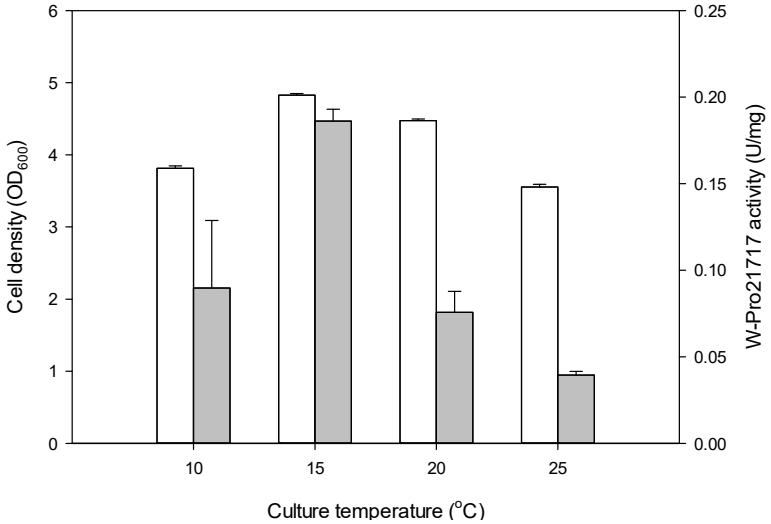

Figure 1. Effect of culture temperature on cell density and W-Pro21717 yield. Cells were grown in modified Marine Broth medium for 72 h and cell density (white bar) and W-Pro21717 activity (gray bar) was measured. Error bars represent the standard deviation of three replicates.

3.2. Effect of temperature on W-Pro21717 activity

W-Pro21717 activity was measured at various temperatures and compared with the activity of a mesophilic protease from *Bacillus licheniformis*. The proteolytic activity was highest at 40°C for both W-Pro21717 and the mesophilic protease (Fig. 2). Compared to 100% activity at 40°C, W-Pro21717 exhibited relative activities of 32% at 10°C and greater than 10% even at 0°C. The mesophilic protease from *B. licheniformis* showed relative activities of 9% and 3% at 10°C and 0°C, respectively. The activity of W-Pro21717 at 60°C drastically decreased to 27% of maximal value, whereas the mesophilic protease retained greater than 90% of its maximum activity. Psychrophilic bacteria never have to endure temperatures higher than 30°C in their environments, and so their enzymes show maximal activity at low temperatures (FELLER & al. [13]).

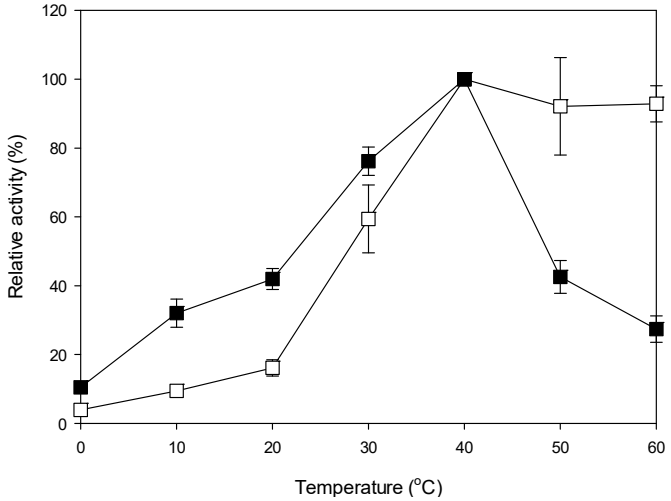

Figure 2. Effect of temperature on protease activity.
Temperature dependence of W-Pro21717 (filled square) and mesophilic protease from *Bacillus licheniformis* (empty square) was determined at various temperatures ranging from 0°C to 60°C for 10 min. Error bars represent the standard deviation of three replicates.

3.3. Thermal stability of W-Pro21717

To determine thermal stability of the protease, both W-Pro21717 and the mesophilic protease were incubated at various temperatures (0−60°C) for 1 h and then relative activity was calculated. We observed a reduction in W-Pro21717 activity at 30°C after 1 h (Fig. 3), whereas activity of the mesophilic protease decreased after 1 h at 40°C. W-Pro21717 retained only 13% of the original activity after 1 h of incubation at 50°C, whereas mesophilic protease retained 63% of its original activity under exactly the same conditions. Generally, an increase in temperature results in an increase in the enzyme reaction rate. However, at temperatures exceeding the optimum condition, the enzyme undergoes inactivation and denaturation. When the Antarctic protease W-Pro21717 was incubated for 1 h at different temperatures, it proved to be significantly more heat-labile than the commercial mesophilic protease.

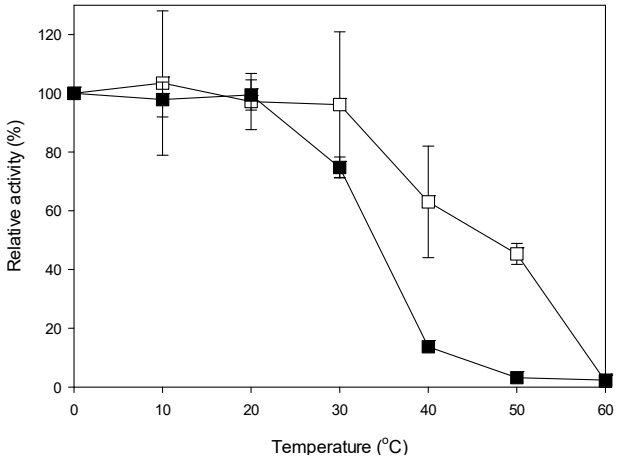

Figure 3. Effect of temperature on protease stability. Thermal stability of W-Pro21717 (filled square) and mesophilic protease from *Bacillus licheniformis* (empty square) was measured after a 1-h incubation at various temperatures. Error bars represent the standard deviation of three replicates.

3.4. Determination of the half-life of W-Pro21717

The proteases were incubated at 40°C for 3 h and enzyme activity was measured at the time points 0, 0.2, 0.5, 0.9, 1.9, and 3 h. The predicted deactivation equation for W-Pro21717 was $y = 100e^{-1.9194x}$, where y is retained activity and x is incubation time (h) at 40°C. The half-life for W-Pro21717 was estimated to be 0.36 h and the half-life of mesophilic protease was 1.0 h, which was 2.8 times longer than that of W-Pro21717 (Fig. 4). These results indicated that W-Pro21717 was a typical cold-active enzyme with relatively high activity at low temperatures and low thermal stability (FELLER & al. [13]).

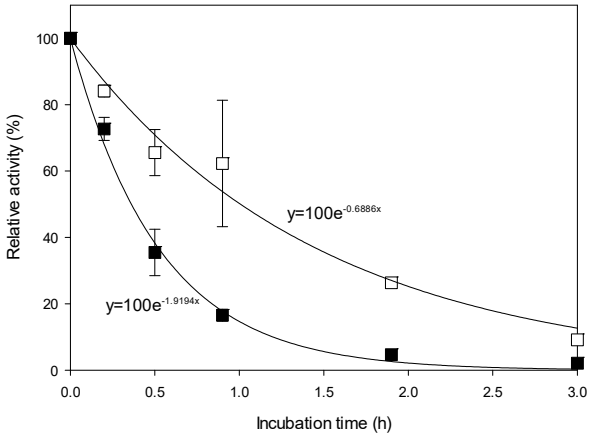

Figure 4. Half-life of W-Pro21717. Deactivation profile of W-Pro21717 (filled square) and mesophilic protease from *Bacillus licheniformis* (empty square) at 40°C for 3 h. Error bars represent the standard deviation of at least three samples taken from a single run.

4. Conclusions

To the best of our knowledge, this study is the first to investigate the effects of temperature on the production, activity, and stability of the cold-active protease W-Pro21717 isolated from *P. arctica*. These results may have a practical application in the production and characterization of cold-active enzymes.

Acknowledgements

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