Cold-Active Enzymes from Cold-Adapted Bacteria

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ABSTRACT: The properties of amylase, lipase and protease, excreted by newly isolated bacteria from snow-covered soil, salmon intestine and crab intestine, have been investigated. One amylase, one lipase, and three proteases have been characterized by shifts in their apparent optimal activities toward low temperatures and by reductions in their activation energy values. The discovered enzymes were rapidly inactivated at temperatures above the optimum (30 to 40°C). These results suggest that the enzymes are cold-active. The best cold-active protease producer, isolated from salmon intestine, has been identified as *Flavobacterium balustinum* by the analysis of 16S rRNA. The optimal growth temperature of this bacterium was 20°C, but a higher amount of protease activity was present at 10°C.
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KEY WORDS: Activation energy, cold-active amylase, cold-active lipase, cold-active protease, crab intestine, detergent additives, extracellular cold-active enzymes, *Flavobacterium balustinum* P104, psychrotrophic bacterium, salmon intestine.

Psychrotrophs are known to synthesize enzymes with low activation energies and high activities at low temperatures (1–3). These properties confer to cold-active enzymes a greater economical value because enzymatically driven reactions can be carried out within a temperature range of 0–20°C. At these temperatures, homologous enzymes from mesophiles have their catalytic activities drastically reduced (4). In this respect, cold-active enzymes raise considerable interest for both industrial use and fundamental studies. Practical utilization of enzymes from cold-adapted microorganisms would constitute considerable progress toward energy savings. One such application would be the use of enzymes as additives to detergents for cleaning at low temperature. Here, we report the selection of five bacterial strains isolated from cold environments for their capacity to secrete cold-active exoenzymes (lipase, amylase, and protease) into culture medium. The characterizations of a cold-active protease and the source bacterium isolated from salmon intestine are discussed in relation to the cold-adapted properties.

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EXPERIMENTAL PROCEDURES

Bacterial strains. One hundred thirty-two microorganisms were isolated from cold soil (snow-covered) of Ishikawa Prefecture and from intestines of salmon (*Oncorhynchus keta*) and crab (*Chionoecetes opilio*). All isolates showed visible growth at 10°C after 3 d.

Identification. After growing at 20°C for 72 h, the characteristics of strain P104 were determined with a scanning electron microscope (S-4500; Hitachi, Tokyo, Japan). It was also tested for biochemical characteristics by using API 20 E strips (Bio Merieux S.A., Marcy-l'Etoile, France) at 20°C for 48 h. Further identification was achieved by determination of the 16S rRNA gene.

Cultivation. The microorganisms were cultivated in a pH neutral medium, composed of polypepton (0.5%), yeast extract (0.25%), and $MgSO_4 \cdot 7H_2O$ (0.02%) in distilled water. Casein sodium (0.5%) or soluble starch (1%) or olive oil (1%) and sodium deoxycholate (0.3%) were further added for producing protease, amylase, and lipase, respectively. Aliquots (25 mL) of the medium in 100-mL flasks were inoculated with 1% of subcultures, grown in the same medium, and incubated for 72 h at 10°C on a rotary shaker with 150 rpm. For determining the effect of temperature on growth and protease excretion, strain P104 was incubated at 10, 20, and 30°C. Growth was monitored by measuring the optical density at 660 nm with a DU 640 spectrophotometer (Beckman, Fullerton, CA). Cell-free supernatant fluids, obtained by centrifugation at 17,000 × *g* and 4°C for 15 min, were assayed for enzyme activity.

Protease activity assay. Proteolytic activity was measured by the digestion of azocasein (5). The supernatant (50 µL) was incubated with 300 µL of 1% azocasein in 67 mM phosphate buffer, pH 7.0, at 20°C for 30 min. The reaction was stopped by adding 1 mL of 6% trichloroacetic acid. After incubation for 15 min at room temperature, the mixture was centrifuged (17,000 × *g*), and the absorbance of the supernatant was measured at 340 nm. The activity was expressed in relative azocasein digestion units (ACU). One ACU was defined as the increase of 0.001 absorbance units per minute under the described conditions.

Amylase activity assay. Amylase activity was measured by the digestion of soluble starch (6). The calibration curve of iodine reaction was made with soluble starch as a substrate.

The supernatant (500 µL) was incubated with 300 µL of 0.2% soluble starch in 50 mM phosphate buffer, pH 7.0, at 20°C. After 30 min, 50 µL of the reaction mixture was added to 1 mL of 0.1 N iodine solution for determining amylase activity. The absorbance of the solution was measured at 600 nm. The activity was expressed in relative soluble starch digestion units. One soluble starch digestion unit of activity was defined as the digestion of 1 µg of starch per minute and was determined based on absorbance vs. a standard calibration curve.

Lipase activity assay. Lipase activity was measured by the digestion of olive oil (7). The calibration curve was made with oleic acid as substrate. The supernatant (100 µL) was incubated with 300 µL of 0.2% olive oil and 0.07% sodium deoxycholate in 50 mM phosphate buffer, pH 7.0, at 20°C. The reaction was stopped by cooling with ice. The lipase activity was determined by a NEFA C-TEST WAKO (Wako, Osaka, Japan) kit with 50 µL of the reaction mixture. Here, the free fatty acid generated from digestion of olive oil was assayed by the modified method of Shimizu *et al.* (8). The resultant blue color, produced from the disappearance of H_2O_2 in the reaction, was assayed at 550 nm. One lipase unit of activity was defined as the production of 1 µg free fatty acid per minute and was calculated based on absorbance vs. a standard calibration curve.

Effect of temperature on activity of enzymes. For determination of the temperature optimum, the reaction was carried out at various temperatures. Activation energies were calculated from the Arrhenius plots of enzymes (9). Proteases from the isolated bacteria were compared with subtilisin Carlsberg (Sigma, St. Louis, MO). Amylase from the isolated bacterium was compared with the reference enzyme from *Bacillus subtilis* (Wako). Lipase from the isolated bacterium was compared with the reference enzyme from *Candida cylindracea* (Sigma).

RESULTS AND DISCUSSION

Strain characterization. In total, 132 microorganisms were isolated and tested for excretion of protease, amylase, or lipase. Strains P104, P105, and P107 were selected among 36 isolates from salmon intestine as potent producers of protease. Strain A201 was selected among 48 isolates from snow-covered soil as a potent producer of amylase. Strain L306 was selected among 48 isolates from crab intestine as a potent producer of lipase. The best cold-active protease producer, strain P104, isolated from salmon intestine, was further characterized. This bacterium is gram-negative, aerobic, and a yellow pigment producer. It is rod-shaped and has a size of approximately 0.4–0.5 × 1.7–1.9 µm, based on observations with an electron microscope. For biochemical characterization, positive tests were found for urease, indole, oxidase and gelatinase, and negative results were found for all other tests with API 20 E strips (Table 1). The API profile index results suggested that the strain P104 belongs to *Flavobacterium indologenes.* However, the 16S rRNA data analysis (not shown) confirmed that it belongs to *F. balustinum,* which is not included in the API profile index. Therefore, this bacterium was named as *F. balustinum* P104 in this study. We propose classifying the strain P104 as a psychrotroph because of its optimal temperature for growth between 10°C to 20°C and significant growth at 30°C (10).

TABLE 1
Biochemical Characterization of *Flavobacterium balustinum* P104

Test	Result
β-Galactosidase	Negative
Arginine dihydrolase	Negative
Lysine decarboxylase	Negative
Ornithine decarboxylase	Negative
Citrate	Negative
H_2S production	Negative
Urease	Positive
Tryptophan deaminase	Negative
Indole production	Positive
Gelatinase	Positive
Glucose	Negative
D-Mannitol	Negative
Inositol	Negative
D-Sorbitol	Negative
L-Rhamnose	Negative
Sucrose	Negative
D-Melibiose	Negative
D-Amygdalin	Negative
L-Arabinose	Negative
Oxidase	Positive

Effect of temperature on activity of the enzymes. Extracellular proteases, excreted from the three potent protease producer strains, were compared with subtilisin Carlsberg for the effect of temperature on activity (Fig. 1). Proteases from

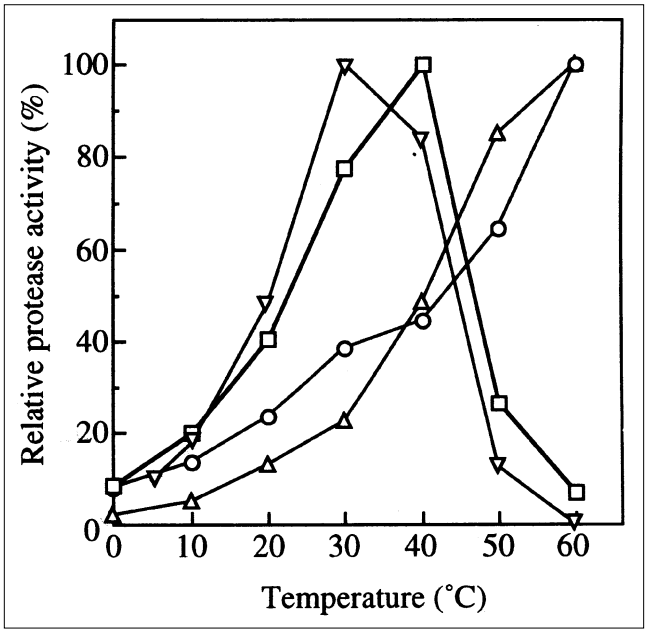

FIG. 1. Effect of temperature on protease activity in culture medium. The reaction was performed with azocasein at pH 7.0 for 30 min. The reference enzyme was subtilisin Carlsberg; □, protease of strain P104;▽, protease of strain P105; ○, protease of strain P107; ○, subtilisin Carlsberg.

strains P104, P105, and P107 showed the highest activities at 40, 30, and 60°C, respectively. For P104 and P105, 40% and 50% of the activities, respectively, could still be detected at 20°C. At 20°C, subtilisin Carlsberg and strain P107 showed only 10 and 25% of the protease activities, respectively, as compared with their maximum activities at 60°C. Subtilisin Carlsberg, produced by mesophilic *Bacillus* sp., and P107 protease showed similar properties for the temperature effect. This could also be due to the presence of high-temperature-active protease in the preparation.

Extracellular amylase, excreted from strain A201, was compared with the reference alpha-amylase of *B. subtilis*. Figure 2 shows that the maximum amylase activity from strain A201 was found at 30°C; however, it was 50°C for the alpha-amylase from *B. subtilis*. Amylase from the strain A201 showed 80% relative activity at 20°C, while the enzyme from *B. subtilis* showed only 20% of the activity at this temperature.

Extracellular lipase, excreted from strain L306, was compared with the reference lipase of *C. cylindracea*. Figure 3 shows that the highest lipase activity from strain L306 was found at 40°C; however, it was 60°C or above for the lipase of *C. cylindracea*. Lipase of strain L306 showed 80% relative activity at 20°C, while the enzyme from *C. cylindracea* showed only 30% of the activity at this temperature.

In this study, they discovered that these enzymes exhibited higher degrees of activity at low temperatures when compared with reference enzymes.

The activation energies of the enzymes. The apparent activation energies of the enzymes were calculated and are shown in Table 2. They followed Arrhenius' Law between 10 and 40°C (data not shown). Activation energies of proteases from strains P104, P105, and P107 were 39.8, 40.1, and 48.3 kJ/mol, respectively, while for the reference enzyme, subtilisin Carlsberg, the value was 53.8 kJ/mol. Activation energies of amylase from the strain A201 and the reference enzyme from *B. subtilis* were 32.5 and 95.7 kJ/mol, respectively. Activation energies of lipase from the strain L306 and the reference enzyme from *C. cylindracea* were 13.8 and 24.3 kJ/mol, respectively. According to these results, the activation energy values of the new enzymes were much lower than the reference enzymes, which confirms that these new enzymes are cold-active.

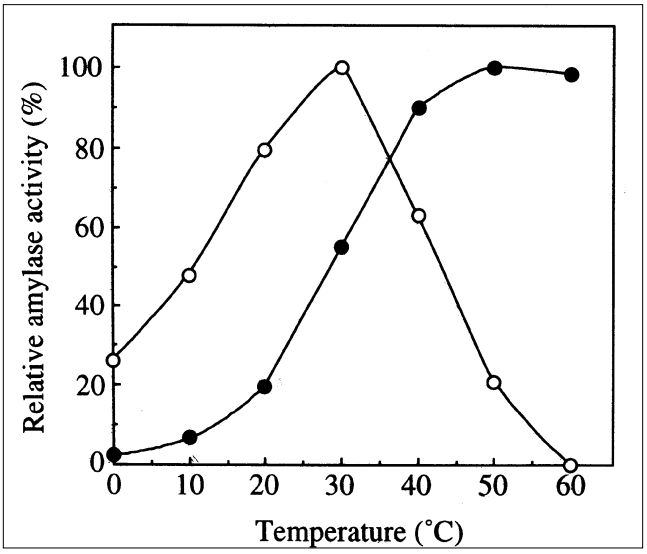

FIG. 2. Effect of temperature on amylase activity in culture medium. The reaction was performed with soluble starch at pH 7.0 for 30 min. The reference enzyme was alpha-amylase from *Bacillus subtilis*; ○, amylase of strain A201; ●, alpha-amylase from *B. subtilis*.

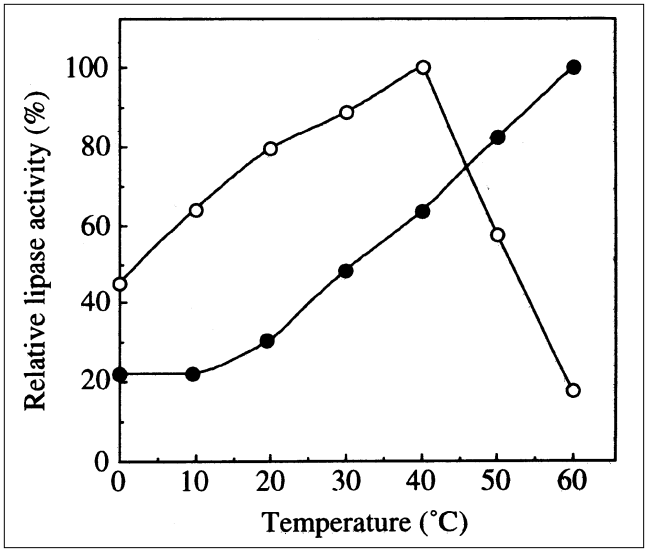

FIG. 3. Effect of temperature on lipase activity in culture medium. The reaction was performed using olive oil emulsion at pH 7.0 for 30 min. The reference enzyme was lipase from *C. cylindracea;* ○, lipase of strain L306; ●, lipase from *C. cylindracea.*

Effect of temperature on the growth of strain P104 and its protease excretion. Figure 4 (A, B, and C) shows that *F. balustinum* P104 grew better at 10 and 20°C than 30°C; the growth rate was highest at 20°C. However, a higher amount of protease activity was present at 10°C than 20°C. Protease activity was negligible at 30°C (Fig. 4C). These low-temper-

TABLE 2
Comparison of Activation Energy Between Reference Enzymes and Extracellular Enzymes from the Isolated Bacteria

Producer	Activation energy (kJ/mol)
Protease	
Strain P104	39.8
Strain P105	40.1
Strain P107	48.3
Bacillus subtilis (subtilisin Carlsberg)	53.8
Amylase	
Strain A201	32.5
B. subtilis	95.7
Lipase	
Strain L306	13.8
Candida cylindracea	24.3

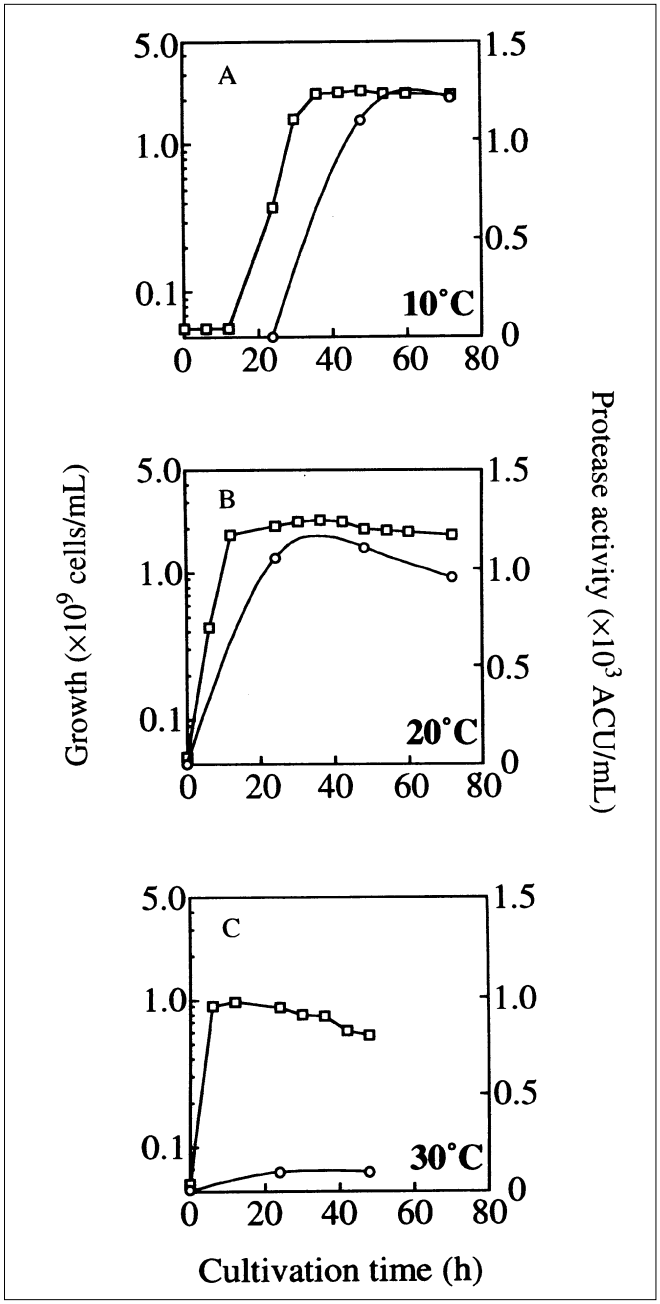

FIG. 4. Relationship between the growth of *Flavobacterium balustinum* P104 and its extracellular protease production at different temperatures: ○, protease activity; □, bacterial growth.

ature properties might be related to the bacterium's ability for surviving in a cold environment (salmon intestine).

The results suggest that these new enzymes are cold-active because the main features of cold-adapted enzymes are lower activation energies and activity at lower temperatures. Only the protease from the strain P107 showed properties similar to subtilisin Carlsberg with respect to the effect of temperature on activity. However, it did exhibit an activation energy lower than that of subtilisin Carlsberg (Table 2).

In this study, extracellular enzymes were isolated and used for subsequent characterization. These extracellular enzymes might contain isozymes. Therefore, the data might result from the cumulative properties of these isozymes. The purification and characterization of the protease from strain P104 are in progress. These results will be reported later.

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