Characterization of a Protease from a Psychrotroph, *Pseudomonas fluorescens* 114

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Received 24 February 1994/Accepted 22 July 1994

A psychrotrophic bacterium isolated from river sediment was identified as *Pseudomonas fluorescens* 114. It grew at 0°C and optimally at 20°C. The bacterium produced a protease with a molecular weight of 47,000, which was stable in the pH range of 5 to 9 and worked optimally between pH 6.5 and 10. Activity was optimal at 35°C and was lost immediately at 50°C and after 5 min at 45°C. At 0, 10, and 20°C, 24, 38, and 57% of optimal activity were observed, respectively.

Extemophiles have adapted to their environments by optimizing their metabolic processes. One class of extremophiles are low-temperature-adapted microorganisms, which grow at temperatures around 0°C (5, 12, 18). Bacteria capable of growing at these low temperatures were first isolated from preserved fish by Forster in 1887 (4). These cold-adapted microorganisms are classified into two categories, psychrophiles and psychrotrophs (psychrotolerants). Morita defined psychrophiles as those bacteria which have an optimal temperature for growth at about 15°C or lower, a maximal temperature for growth at about 20°C or lower, and a minimal temperature for growth at 0°C or lower. Bacteria that grew at temperatures such as 5°C or lower but had higher optimal-growth temperatures were defined as psychrotrophs (12). The metabolic processes in psychrophiles and psychrotrophs have been found to be heat labile (11, 15). Studying the characteristics of enzymes from cold-adapted microorganisms may provide novel ways of understanding thermobiochemistry as well as lead to possible applications of such enzymes in the processing of food and the biotransformation of chemicals at low temperatures.

Numbers of investigations of extracellular enzymes from psychrophiles and psychrotrophs of different origins have been reported (1, 3, 7, 9, 10, 14, 16). Recently, we isolated psychrophilic and psychophilic microorganisms from various cold environments. Two psychrotrophic bacteria, *Vibrio* strain 4-3 and *Vibrio* strain 814-4, isolated from deep-sea sediment samples, were found to produce cold-adapted amylases (7, 8). These enzymes retained significant activity at low temperatures, 40% at 10°C in the case of strain 4-3 and 35% at 10°C in the case of strain 814-4. The psychrophilic nature of these amylases suggested that they may provide useful systems for the biochemical study of thermostability, and it may be possible to apply these enzymes in the processing of starch at low temperatures.

Psychrotrophic microorganisms have been isolated not only from permanently cold environments like the deep sea but also from seasonally cold environments because of their ability to tolerate higher temperatures (13). Psychrotrophs are believed to play an important role in the biodegradation of organic matter in seasonally cold environments like river water. Accordingly, we have screened for cold-adapted enzymes from seasonally cold environments. In the course of this screening, we have isolated from a river sediment sample a psychrotrophic bacterium which produces a protease which is active at low temperatures. This paper reports the isolation, purification, and biochemical characterization of the protease.

The isolation of psychrotrophs was carried out by directly spreading sediment samples on slightly modified Luria-Bertani (LB) agar plates (Bacto Tryptone, 10 g; yeast extract, 5 g; sodium chloride, 10 g; agar, 18 g) and incubating them at 5°C. Bacterial colonies which appeared on the agar plates were purified, and the proteolytic activities of these psychrotrophs were examined by looking for zones of hydrolysis on the modified LB agar plates containing 0.5% casein (Casein in Hammarsten; Merck). From 41 samples collected from river sediments, pond water, and soil covered with snow, 241 different bacterial colonies were formed at 5°C. From a sediment sample collected at the Oppe River, Saitama, Japan (water temperature around 10°C), aerobic psychrotrophic bacteria were isolated on LB agar plates incubated at 5°C. Fifteen bacteria which showed strong proteolytic activities on casein at 4°C were isolated, and one strain was selected for further study. The strain was a gram-negative, motile, and obligatory aerobic rod (2 to 3 by 0.5 μm), and it produced a fluorescent pigment in King's medium B (2% Bacto Peptone, 1% glycerol, 0.15% KH2PO4, 0.15% MgSO4) (2). The bacterium was glucose oxidative, catalase positive, and cytochrome oxidase positive. Its moles percent G+C content was determined by high-pressure liquid chromatography of hydrolyzed nucleotides with a kit provided by Seikagaku Kogyo, Tokyo, Japan, and used according to the manufacturer's protocols. Biochemical characteristics were examined with the NF-18 bacterial identification kit (Nissui Pharmaceutical, Tokyo, Japan). The moles percent G+C content (59.6%), combined with the biochemical characteristics examined, indicated that the strain belonged to the species *Pseudomonas fluorescens*.

Protease activity in culture supernatants of *P. fluorescens* 114 grown in 2 liters of liquid LB medium was observed after 24 h at 20°C. Protease activities were measured by the amount of acid-soluble fragments released from casein at 30°C unless otherwise stated. Culture supernatants (100 μl) were incubated with 500 μl of 1% casein and 400 μl of 80 mM imidazole-HCl buffer (pH 7.8). After incubation, an equal volume of TCA solution (0.11 M trichloroacetic acid, 0.22 M sodium acetate, and 0.12 M acetic acid) was added to the reaction mixture. TCA-insoluble materials were removed by

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TABLE 1. Purification of the protease

<table>
<thead>
<tr>
<th>Protease medium</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (kU)</th>
<th>Specific activity (U/mg of protein)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernatant</td>
<td>1,940</td>
<td>1,785</td>
<td>591</td>
<td>331</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate precipitate</td>
<td>60</td>
<td>130</td>
<td>293</td>
<td>2,264</td>
<td>50</td>
</tr>
<tr>
<td>DEAE Toyopearl 293</td>
<td>116</td>
<td>25</td>
<td>188</td>
<td>7,386</td>
<td>32</td>
</tr>
<tr>
<td>Superdex 200</td>
<td>57</td>
<td>9.1</td>
<td>61</td>
<td>6,645</td>
<td>10</td>
</tr>
</tbody>
</table>

The results of purification are summarized in Table 1. The overall recovery was 10%, with a purification factor of 20. The loss of specific activity in the last gel filtration step was assumed to be caused by autolysis in the concentrated preparation. The purity of the enzyme was confirmed by SDS-PAGE (Fig. 1) and gel filtration chromatography. The molecular weight of the enzyme, deduced by SDS-PAGE and Superdex 200 gel filtration chromatography, was $4.7 \times 10^4$.

The effect of temperature on the activity of the protease is shown in Fig. 2. Enzyme activities at various temperatures were measured by incubating the reaction mixtures in 80 mM imidazole-HCl buffer (pH 7.8) at designated temperatures for 5, 10, and 20 min. The enzyme had an optimal temperature for activity at around 35 to 40°C. At 0, 10, and 20°C, the enzyme maintained 24, 38, and 57% of its activity at the optimum temperature, respectively. Activity was completely and immediately lost at 50°C because of thermal inactivation. Between 10 and 20 min, there was a greater increase in protease activity at 35°C than at 40°C, perhaps indicating greater stability at this lower temperature. At 45°C, activity decreased after 5 min of incubation.

Enzyme activity at various pH values was observed at 30°C for 10 min in reaction mixtures with 80 mM buffer at various pHs (acetate buffer between pH 5.0 and 5.5, potassium phosphate buffer between pH 5.5 and 8.0, imidazole-HCl buffer between pH 6.5 and 8.0, and glycine-NaOH buffer between pH 8.5 and 13.0). Protease activity was optimal between pH 6.5 and 10 (Fig. 3).

The effects of metal ions and chemicals on enzyme activity were measured by adding designated concentrations of substances to reaction mixtures and incubating them for 20 min at 30°C. Enzyme activity was not affected by protease inhibitors,
such as 10 mM phenylmethylsulfonyl fluoride or 10 mM iodoacetate. In contrast, 10 mM EDTA partially inhibited activity (63% of activity retained). These results suggested that this enzyme was a neutral metalloprotease. Protease activity was also inhibited by 1 mM Ni\(^{2+}\) (38% of activity retained) and Cu\(^{2+}\) (30% of activity retained), although it was only slightly affected by the presence of 0.1 mM Hg\(^{2+}\) (85% of activity retained). In 3 mM SDS, only 20% of enzyme activity was retained, but in the neutral detergent dodecylbenzenesulfonate almost full activity was retained.

Similarly low optimum temperatures are observed with other proteases from psychrophiles and psychrotrophs. An alkaline protease from a psychrophile, *Escherichia freundii*, had an optimum temperature of 25°C at pH 10, which is slightly lower than that of the protease of *P. fluorescens* 114 (14). However, a yeast isolated in Antarctica, *Candida humicola*, produced a protease with an optimum temperature of 37°C (17), and a psychrophile, *Xanthomonas maltophilia*, produced a protease with an optimum temperature of 50°C (10).

Because of their relatively high activities at low temperatures, psychrophiles and psychrotrophs are of great scientific interest. Furthermore, enzymes that are active at low temperatures, such as the protease discussed in this paper, may have biotechnological and industrial importance (6).

We are grateful to R. Y. Morita, W. D. Grant, and T. McGenity for critically reading the manuscript. We also thank Teruhiko Akiba and Shizuko Horike for helpful discussions.

This work was partially supported by a biodesign research grant of the Institute of Physical and Chemical Research to T.K.

REFERENCES


