Partial Purification and Characterization of Thermostable Esterase from the Hyperthermophilic Archaeon Sulfolobus solfataricus

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Abstract A thermostable esterase from the hyperthermophilic archaeon Sulfolobus solfataricus was partially purified 590-fold with 16.2% recovery. The partially purified esterase had a specific activity of 29.5 µmol min⁻¹ mg⁻¹ when the enzyme activity was determined using p-nitrophenyl butyrate as a substrate. The apparent molecular weight was about 100 kDa, while the optimum temperature and pH for esterase were 75°C and 8.0, respectively. The enzyme showed high thermal stability and solvent tolerance in comparison to its mesophilic counterpart. The enzyme also showed chiral resolution activity for (S)-ibuprofen, indicating that Sulfolobus solfataricus esterase can be used for the production of commercially important chiral drugs.

Keywords: Sulfolobus solfataricus, thermostable esterase, ibuprofen resolution

Thermophilic enzymes have potent biotechnological advantages for ensuring their stability under extreme environments such as high temperature, extreme pH, and low water content. A number of industrially available enzymes from thermophilic microorganisms have been studied and purified. Some of them are currently used in industrial processes [1]. Among thermophilic organisms, hyperthermophilic archaea are expected to benefit industrial processes due to their superior stability in harsh external conditions. Therefore, efforts were taken to utilize hyperthermophilic enzymes in industrial applications.

Esterase is a promising enzyme used in many industrial applications, such as synthesis of esters and resolution of chiral drugs [2]. Enzymatic resolution of chiral drugs is considered an easy and time-saving process as compared to chemical asymmetric synthesis. There are many examples of the use of mesophilic esterase in such stereochemistry, but only a few examples of thermostable esterase being employed in stereochemical industry.

The thermostable esterases that have been purified and characterized so far were obtained from Sulfolobus acidocaldarius [3], Bacillus acidocaldarius [4], and Bacillus stearothermophilus [5]. Among these, esterase from S. acidocaldarius is the only one derived from hyperthermophiles. Moreover, the application of hyperthermophilic esterase in chiral resolution has not yet been reported. In this study, we performed partial purification and characterization of a thermostable esterase from the hyperthermophilic archaeon Sulfolobus solfataricus. S. solfataricus (DSM 1617) was grown at 78°C and pH 3.0 in a 2.5-L fermenter. Esterase was purified with an FPLC (Pharmacia, Sweden) system. To purify esterase, wet frozen cells were thawed at 4°C in a 0.1 M Tris-HCl (pH 7.0) buffer and sonicated for cell lysis. Cell debris was removed by centrifugation (10,000 g, 30 min, 4°C). Ammonium sulfate was slowly added to a final saturation level of 80%. After centrifugation at 40,000 g for 3 min, the resulting precipitate was dissolved in a 20 mM-potassium phosphate buffer (pH 6.8) and dialyzed overnight against 10 liters of the same buffer. The dialyzed sample was loaded to a hydroxyapatite column (2.6 × 47 cm: Bio-Rad, California, U.S.A.) equilibrated with a potassium phosphate buffer (20 mM, pH 6.8). The enzyme was eluted with a linear gradient (0.02-0.5 M) of the potassium phosphate buffer (pH 6.8). After centrifugation (40,000 g, 3 min, 4°C), the precipitate was dissolved in a 50 mM sodium phosphate buffer (pH 7.0) containing 1 M NaCl.

Hydrophobic interaction chromatography was performed at 50 mL Phenyl-Sepharose (2.6 × 12 cm: Pharmacia, Sweden) equilibrated with a 50 mM sodium phosphate buffer (pH 7.0) containing 1 M NaCl. The enzyme was eluted with a linear gradient of ethylene glycol (0-50% v/v) in a 50 mM sodium phosphate buffer (pH 7.0), then with 50% ethylene glycol in a 50 mM sodium phosphate buffer (pH 7.0) successively. The active fraction was collected and concentrated with an Amicon (Beverly, U.S.A.) ultrafiltration cell (centriprep10, centricon30). To determine the molecular weight, a Superose 12 column (Pharmacia, Sweden) was calibrated using a molecular weight marker. The apparent molecular weight was calculated by interpolating a linear plot of log (molecular weight) versus V/Vo ratio.

Esterase activity was determined by using two meth-
Table 1. Purification of esterase from *S. solfataricus*

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>2350.8</td>
<td>117.5</td>
<td>0.05</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>994.5</td>
<td>79.5</td>
<td>0.08</td>
<td>6</td>
<td>67.7</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>4.8</td>
<td>25.3</td>
<td>5.3</td>
<td>106</td>
<td>21.6</td>
</tr>
<tr>
<td>Superose 12</td>
<td>0.65</td>
<td>19.0</td>
<td>29.5</td>
<td>590</td>
<td>16.2</td>
</tr>
</tbody>
</table>

ods. (A) *p*-Nitrophenyl butyrate method: *p*-Nitrophenyl butyrate was dissolved to 10 mM in acetonitrile for stock solution preparation. A substrate stock solution was prepared by mixing stock solution, ethanol, and 50 mM Tris/HCl (pH 7.0) with a ratio of 1:4:95, and 50 µL of this substrate stock solution was added to the 4.9 mL of 50 mM Tris/HCl (pH 7.0). The reaction was started by adding a 50 µL of enzyme solution to the buffered substrate solution. The release of *p*-nitrophenol by enzymatic hydrolysis was monitored by the absorbance change at 405 nm continuously and background hydrolysis due to nonenzymatic reaction. (B) 4-Methylumbelliferyl acetate method: 100 mM of 4-methylumbelliferyl acetate dissolved in dimethyl sulfoxide (50 µL) was added to the 4.4 mL of a 50 mM Tris-HCl buffer (pH 7.0) preincubated at 75°C. The reaction was started by adding 50 µL of enzyme solution and stopped by adding 0.5 mL citric acid (0.3 M) after 3 min. The absorbance change was measured at 354 nm. Protein concentration was determined according to Bradford [6] using bovine serum albumin as a standard.

To study the effect of pH on enzyme activity, a 50 mM succinic acid-NaOH buffer (pH 3.8 to 5.7), a 50 mM sodium phosphate buffer (pH 5.9), a 50 mM Tris-HCl buffer (pH 6.5 to 7.7), and a 50 mM boric acid-NaOH buffer (pH 8.2 to 9.6) were used. To investigate the effect of organic solvents on enzyme activity, water-miscible organic solvents (methanol, ethanol, acetonitrile, tetrahydrofuran, dimethylformamide and dioxane) were used. Each organic solvent was dissolved in a 50 mM Tris-HCl buffer (pH 7.0) at a concentration range of 0 to 50% (v/v).

The correlation between cell growth and enzyme activity revealed that the esterase activity of *S. solfataricus* was associated with cell growth. Therefore, cells were harvested at a late stationary phase to obtain the maximum amount of esterase and cell biomass. After three steps of chromatography, hydroxyapatite, Phenyl-Sepharose and Superose 12, the thermostable esterase was partially purified approximately 590 fold with the specific activity of 29.5 µmol min⁻¹ mg⁻¹ and 16.2% recovery (Table 1). The most effective step was the use of a Phenyl-Sepharose column, which elevated specific activity approximately 66 fold. There was no detection of activity in a first gradient of Phenyl-Sepharose. The activity peak was obtained by decreasing polarity in a second gradient, *i.e.* by increasing ethylene glycol concentration up to 50%. This suggests that esterase from *S. solfataricus* has very hydrophobic surface. The apparent molecular weight of a native enzyme determined by gel filtration was about 100 kDa (Fig. 1).

*Fig. 1.* Molecular mass determination of esterase from *S. solfataricus* by gel-filtration chromatography on a Superose 12 column. Marker proteins: ribonuclease (13.7 kDa), chymotrypsinogen A (25 kDa), albumin (67 kDa), aldolase (158 kDa). The arrow indicates *S. solfataricus* esterase.

*Fig. 2.* Arrhenius plot of temperature dependence. Crude enzyme and 50 mM Tris/HCl (pH 7.0) were incubated at the indicated temperatures. Esterase activity was obtained by using *p*-nitrophenyl butyrate as a substrate.

*S. solfataricus* has very hydrophobic surface. The apparent molecular weight of a native enzyme determined by gel filtration was about 100 kDa (Fig. 1).

The *S. solfataricus* esterase showed an optimum temperature of 75°C, which agrees with the optimum growth temperature of *S. solfataricus* [7]. Thermal inactivation of the enzyme appeared above 75°C. Fig. 2