Biotechnol. Bioprocess Eng. 2000, 5: 53-56

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

Young Mi Chung, Chan B. Park, and Sun Bok Lee*

Department of Chemical Engineering, Pohang University of Science and Technology, San 31, Hyoja Dong, Pohang 790-784, Korea

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 *S. solfataricus* esterase can be used for the production of commercially important chiral drugs.

Keywords: *S. 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 thermophilic 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 *Solfolobus solfataricus*.

* Corresponding author
Tel : +82-562-279-2268 Fax : +82-562-279-2699
e-mail: sblee@postech.ac.kr

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, Califonia, 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_e/V_0 ratio.

Esterase activity was determined by using two meth-

Table 1. Purification of esterase from *S. solfataricus*

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

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

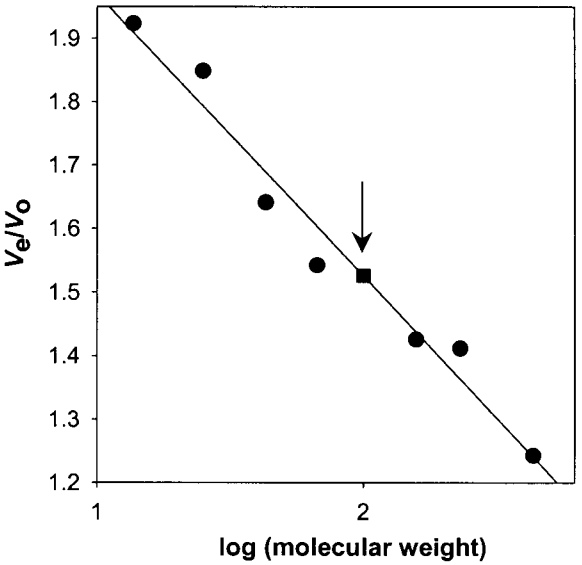

Fig. 1. Molecular mass determination of esterase from *S. solfataricus* by gel-filtration chromatography on a Superose 12 column. Marker proteins: ribonuclease (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), albumin (67 kDa), aldolase (158 kDa). The arrow indicates *S. solfataricus* esterase.

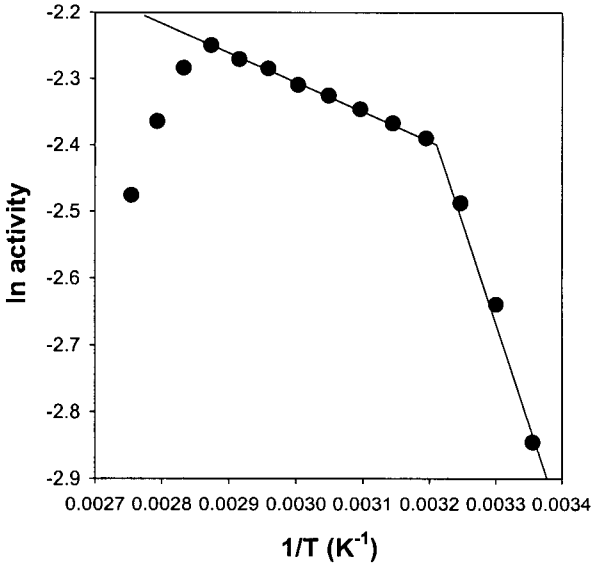

Fig. 2. Arrhenius plot of temperature dependence. Crude enzyme and 50 mM Tris/HCl (pH 7.0) were incubated at the indicated temperatures. Esterase activity was obtained by using *p*-nitrophenyl butyrate as a substrate.

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

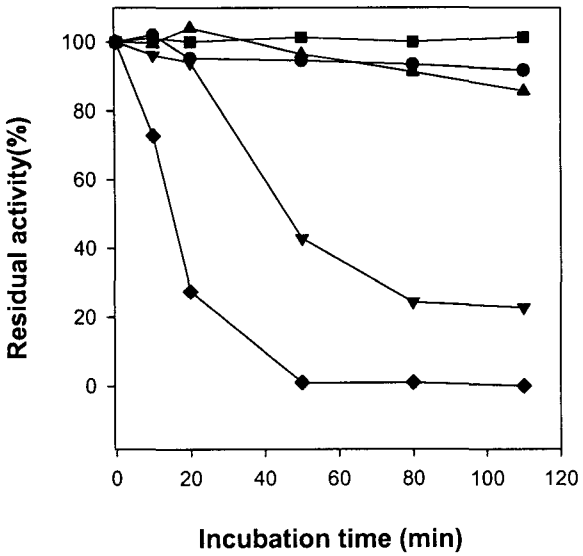

Fig. 3. Thermal stability of *S. solfatricus* esterase as a function of temperature. Crude enzyme in 50 mM Tris/HCl (pH 7.0) was incubated at 80°C (■), 85°C (●), 90°C (▲), 95°C (▼) and 100°C (◆). Samples were chilled in ice and assayed for enzyme activity using *p*-nitrophenyl butyrate.

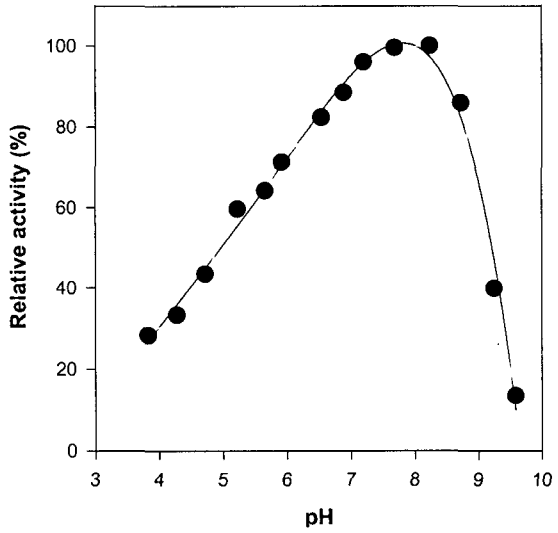

Fig. 4. Effect of pH on enzyme activity. The activity of esterase was obtained by using 4-methylumbelliferyl acetate as a substrate at various pH levels. Buffers for each pH level were a 50 mM succinic acid-NaOH buffer (pH 3.8-5.7), a 50 mM sodium phosphate buffer (pH 5.9), a 50 mM Tris-HCl buffer (pH 6.5-7.7), and a 50 mM boric acid-NaOH buffer (pH 8.2-9.6).

shows the temperature dependency of enzyme activity on temperature as an Arrhenius plot. A break point appeared in the temperature dependency around 40°C. The activation energy for hydrolysis of *p*-nitrophenyl butyrate was about 54.3 kJ/mol at 25-40°C and 8.2 kJ/mol at 40-75°C. A sudden decrease of activation energy at 40°C suggests that the enzyme may experience some increase in structural flexibility above 40°C.

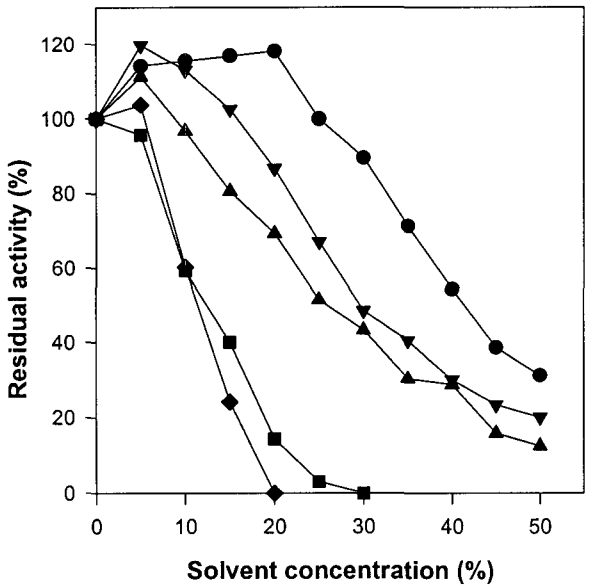

Fig. 5. Effect of organic solvents on enzyme activity. Crude enzyme was added to a 50 mM Tris-HCl buffer (pH 7.0) containing various concentrations of organic solvents, and esterase activity was obtained by using 4-methylumbelliferyl acetate as a substrate. Organic solvents used for reactions were methanol (●), ethanol (▼), acetonitrile (■), tetrahydrofuran (◆) and dimethylformamide (▲).

The *S. solfataricus* esterase showed remarkable thermostability, maintaining initial activity for a lengthy period up to 90°C (Fig. 3). At temperatures higher than 95°C, the activity decreased rapidly: the half-life of esterase at 95 and 100°C was 45 and 15 min, respectively. The optimum pH for enzyme activity was about pH 8.0 (Fig. 4), which shows that intracellular pH is different than extracellular pH. In other words, intracellular pH seems to be neutral, although *S. solfataricus* grows under acidic conditions (pH 3.0).

Fig. 5 shows the effects of organic solvents on enzyme activity. It can be seen that this thermostable enzyme is also stable at high concentrations of polar organic solvents, such as ethanol and methanol as compared to other mesophilic counterparts. The increase of enzyme activity at low concentrations of organic solvents is remarkable. This may be explained by the increase of enzyme flexibility in low concentrations of denaturants such as organic solvents [8-10].

We also found that *S. solfataricus* esterase has a high enantiomer selective property for ibuprofen. Ibuprofen (2-(*p*-isobutylphenyl) propionic acid), a nonsteroidal anti-inflammatory drug, is a commonly used chiral drug biologically active form in (S)-form. Through the enzymatic hydrolysis of ibuprofen esters using *S. solfataricus* esterase, we were able to obtain optically pure (S)-ibuprofen.

In this experiment, (R, S)-ibuprofen methyl ester,

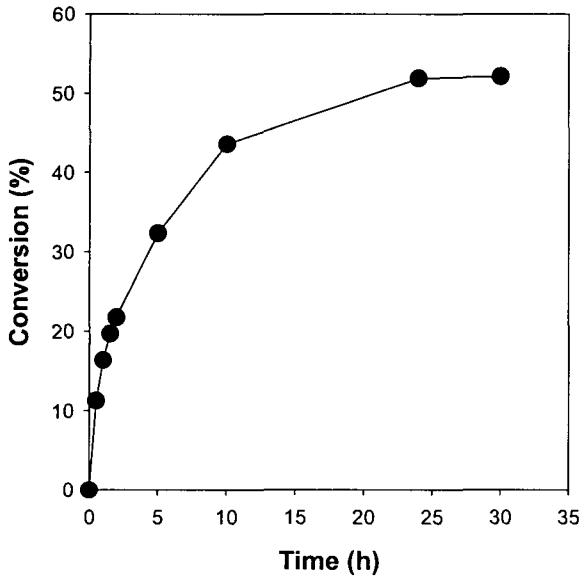

Fig. 6. Time course of ibuprofen conversion. Crude enzyme and (R, S)-ibuprofen methyl ester (50 mM) were incubated at 75°C. Reaction mixtures were extracted by cyclohexane for HPLC analysis.

Which was synthesized from ibuprofen and methanol as described elsewhere [8,9], was added to 10 mL of crude enzyme solution in 50 mM Tris-HCl (pH 7.0) with a concentration of 50 mM. The reaction was performed at 75°C with tightly sealed vessels. After each time interval, the reaction mixtures were withdrawn and mixed with equal volumes of cyclohexane. For HPLC analysis, this reaction mixture was extracted by the addition of H_2SO_4 to decrease the pH level. Ibuprofen produced by enzymatic hydrolysis and unreacted ester were analyzed quantitively by HPLC (Knauer, Germany) using a C_{18} column (Supelco, U.S.A.). The eluent was prepared by mixing deionized water and acetonitrile with a ratio of 2:8 and adjusted to pH 3.1 with phosphoric acid. The flow rate of eluent was 1 mL/min; peaks were detected at 246 nm. The optical purity of ibuprofen produced by enzymatic hydrolysis was analyzed with a Chirex™ column (Phenomenex, U.S.A.) at 230 nm with a flow rate of 0.5 mL/min; the eluent composition was 5 mM-ammonium acetate in methanol.

When (R, S)-ibuprofen methyl ester was hydrolyzed using *S. solfataricus* esterase, about 50% of ibuprofen methyl ester was converted to ibuprofen (Fig. 6). This suggests that only one form of racemate may have participated in hydrolysis reaction. To confirm the chiral resolution activity of *S. solfataricus* esterase, the optical purity of produced ibuprofen was measured. The product obtained at 30-h reaction was pure (S)-ibuprofen (purity>99%). Therefore, it appears that *S. solfataricus* esterase has S-form specificity for ibuprofen, and that such chiral activity of this enzyme may have some significance in the pharmaceutical industry.

Acknowledgements This work was supported by the Korean Ministry of Science and Technology.

REFERENCES

[1] Cowan, D. A. (1992) Biotechnology of the archae. *Trends Biotechnol.* 10: 315-323.
[2] Margolin, A. L. (1993) Enzyme in the synthesis of chiral drug. *Enzyme Microb. Technol.* 15: 266-280.
[3] Sobeck, H. and H. Gorisch (1988) Purification and characterization of a heat-stable esterase from the thermoacidophilic archaebacterium *Sulfolobus acidocadaricus*. *Biochem. J.* 250: 453-458.
[4] Manco, G., S. D. Gennaro, M. D. Rosa, and M. Rossi (1994) Purification and characterization of a thermostable carboxylesterase from the thermoacidophilic eubacterium *Bacillus acidocaldarius*. *Eur. J. Biochem.* 221: 965-972.
[5] Matsunaga, A., N. Koyama, and Y. Nosoh (1974) Purification and properties of esterase from *Bacillus stearothermophilus*. *Arch. Biochem. Biophys.* 160: 504-513.
[6] Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254
[7] Brock, T. D., K. M. Blrock, R. T. Belly, and R. L. Weiss (1972) *Sulfolobus*: a new genus of sulfur-oxidizing bacteria living at low pH and high temperature. *Arch. Mikrobiol.* 84: 54-68.
[8] Guagliardi, A., G. Manco, M. Rossi, and S. Bartolucci (1989) Stability and activity of a thermostable malic enzyme in denaturants and water-miscible organic solvents. *Eur. J. Biochem.* 183: 25-30.
[9] Sundaram, T. K., I. P. Wright, and A. E. Wilkinson (1980) Malate dehydrogenase from thermophilic and mesophilic bacteria. Molecular size, subunit structure, amino acid composition, immunochemical homology, and catalytic activity. *Biochemistry* 19: 2017-2022.
[10] Veronese, F. M., E. Boccu, O. Schiavon, C. Grandi, and A. Fontana (1984) General stability of thermophilic enzymes: studies on 6-phosphogluconate dehydrogenase from *Bacillus stearothermophilus* and yeast. *J. Appl. Biochem.* 6: 39-47.

[Received January 5, 2000; accepted February 11, 2000]