Purification and Characterization of Thermostable Purine Nucleoside Phosphorylase of *Bacillus stearothermophilus* JTS 859

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Received March 8, 1989

A thermostable purine nucleoside phosphorylase has been purified more than 800-fold from *Bacillus stearothermophilus* JTS 859. The enzyme had a molecular weight of 68,000 consisting of 2 identical subunits (M_w , 34,000). The isoelectric point of the enzyme was 4.7. The enzyme did not contain cysteine. The optimal pH of the enzyme reaction was from 7.5 to 11.0. The Michaelis constants for inosine, guanosine, 2'-deoxyinosine, and 2'-deoxyguanosine were 0.22, 0.14, 0.20, and 0.10 mM, respectively. The optimal temperature of the reaction was 80°C. The half-life of the enzyme was 16 hr in 20 mM potassium phosphate and 1 mM inosine (pH 7.0) at 80°C, and no decrease of the enzyme activity was observed at least for the first 30 hr at 70°C.

Purine nucleoside phosphorylase (PUNP) (EC 2.4.2.1) catalyzes the reversible phosphorylase of ribonucleoside and 2'-deoxyribonucleoside derivatives of hypoxanthine, guanine, and xanthine as follows:

As the substrate specificity of the enzyme is not strict, it has been used to prepare nucleoside analogues such as 6-(dimethylamino)-9-(β -D-2'-deoxyribosyl)purine, α -L-lyxosylhypoxanthine, virazole, and so on.^{1~3)} PUNP derived from methophiles has been used for this purpose.

The use of thermostable enzymes has several advantages.⁴⁾ Enzymatic reactions can be done at high temperatures, and reaction rates are also high. Further, higher yields of enzyme would result due to greater enzyme stability at ordinary temperatures. In this study, purification was done at room temperature, except for dialysis. Thermostable enzyme reactors are tolerant of bacterial contamination due to the high operation temperature. This is important for practical application because the usual

enzyme reactors tend to be contaminated by bacteria.

We reported in our previous paper that a thermophile, *Bacillus stearothermophilus* JTS 859, produces a thermostable PUNP.⁵⁾ Its half-life in the crude enzyme solution was 7200 hr and 150 hr at 63°C and 70°C, respectively. This paper describes the purification of the thermostable PUNP from *B. stearothermophilus* JTS 859 and its properties.

Materials and Methods

Microorganism. *Bacillus stearothermophilus* JTS 859, which produced a thermostable purine nucleoside phosphorylase, was used as the enzyme source.

Chemicals. Inosine was purchased from Yamasa Chemicals (Tokyo). Peptone A and yeast extract were purchased from Kyokuto Co. (Tokyo).

Culture conditions. Medium, containing (g/l of water) Peptone A 20, yeast extract 10, glucose 3, inosine 1, and NaCl 3 (pH 6.0) was used for cultivation. Spores (3.2×10^7) on a slant were transferred to 2 l of the medium in a jar fermentor. Cultivation was done at 680 rpm of two flat-blade impellers having 4 upper and 6 under blades (impeller diameter 60 mm) with 1.5 vvm aeration at 65°C for 8 hr. The pH of the medium was maintained from 5.9

to 6.2 by a pH stat during cultivation. The cells were harvested by centrifugation ($10,000 \times g$, 4°C , 15 min).

Enzyme assay. The standard reaction mixture consisting of 20 mM inosine, 20 mM potassium phosphate, and 50 μl of enzyme solution in a total volume of 1 ml (pH 7.0) was incubated at 60°C for 20 min. Fifty μl of the reaction mixture was transferred to 950 μl of MeOH to stop the reaction. The hypoxanthine formed was measured by HPLC.

Analysis. The bases and nucleosides were identified and measured by HPLC. The operating conditions were: column, Chemcosorb 5-ODS-H ($\phi 4 \times 300$ mm) (Chemco Scientific Co, Ltd); column temperature 38°C ; solvent, water–MeOH = 95 : 5 (v/v); flow rate, 0.8 ml/min; detector, UV monitor 254 nm.

Protein assay. Protein was measured by the method of Lowry *et al.* using bovine serum albumin as a standard.⁶⁾

Measurement of molecular weight. The operating conditions of gel filtration were: column, TSK gel G3000SW (ϕ 7.5 $\times$ 600 mm) (Tosoh Co, Tokyo); solvent, 200 mM potassium phosphate (pH 6.8); flow rate, 0.5 ml/min; detector, UV monitor 280 nm. Ribonuclease A (M_w 13.7 k), chymotrypsinogen (M_w 25 k), ovalbumin (M_w 45 k), bovine serum albumin (M_w 67 k), catalase (M_w 240 k), and ferritin (M_w 440 k) were used as standards.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was done by the method of Laemmli⁷⁾

Amino acid analysis. The enzyme (20 μg) was hydrolyzed in 6 N HCl containing 4% thioglycolic acid (400 μl) at 110°C for 22 hr and 70 hr. Amino acid analysis was done with a Hitachi 835 amino acid analyzer.

Results

Purification

All purification procedures were at room temperature except dialysis (4°C). The 20 mM potassium phosphate buffer (pH 7.0) used for the purification is referred as “the buffer” in the text.

Step 1. Cell free extract. Cells (160 g) suspended in the buffer (500 ml) were collapsed by a Dyno-mill KDL type (Willy A. Bachofen AG., Basel, Switzerland) for 3.6 min. The buffer was added to the resulting suspension up to 1400 ml. After centrifugation ($8300 \times g$, 4°C , 20 min), 1300 ml of supernatant was obtained. The precipitate was suspended in the buffer (500 ml) and the suspension was treated by the

Dyno-mill (5 min) again. The buffer was added to the resulting suspension up to 1030 ml. After centrifugation ($8300 \times g$, 4°C , 20 min), the supernatant obtained was combined with the supernatant above.

Step 2. Heat treatment. The combined supernatant was kept at 63°C for 1 hr with gentle agitation. After centrifugation ($8300 \times g$, 4°C , 20 min) 2130 ml of supernatant was obtained.

Step 3. Acetone treatment. After adding a solution (-10°C) consisting of acetone (200 ml) and the buffer (200 ml) to the heat-treated supernatant, 1.8 l of acetone (-10°C) was added to it. This was kept at $5 \sim 10^{\circ}\text{C}$ for 15 min with gentle agitation. After removing the precipitate by centrifugation ($9000 \times g$, 4°C , 5 min), 2.5 l of acetone (-10°C) was added to the supernatant. It was kept at $5 \sim 10^{\circ}\text{C}$ for 15 min with gentle agitation. After centrifugation ($9000 \times g$, 4°C , 5 min), the collected precipitate was dissolved in the buffer (800 ml).

Step 4. Acid treatment. The enzyme solution was adjusted to pH 4.5 with 1 M phosphoric acid and it was kept at 4°C for 1 hr with gentle agitation. After centrifugation ($8900 \times g$, 4°C , 1 hr), the pH of the supernatant was adjusted to pH 7.0 with 1 M KOH.

Step 5. DEAE-Toyopearl 650M chromatography. The enzyme solution (800 ml) was applied on a column of DEAE-Toyopearl 650 M (200 ml) (Tosoh Co., Tokyo). The enzyme was eluted with the buffer (2 l) and with 1 l each of buffer supplemented with 50, 100, 150, and 200 mM NaCl. The enzyme activity was found in two different fractions. The enzyme, eluted with the buffer supplemented with 50 mM NaCl (500 ml), was named PUNPI and the purification and characterization of PUNPI is described in this report. The other enzyme, eluted with the buffer supplemented with 200 mM NaCl (500 ml), was named PUNPII, which will be reported on elsewhere.

Step 6. Second DEAE-Toyopearl 650M chromatography. The enzyme solution, adjusted to pH 8.0 with 1 M KOH, was applied on a column of DEAE-Toyopearl 650 M (400 ml) (Tosoh Co.). The enzyme was eluted with

a linear NaCl gradient from 50 mM to 200 mM in the buffer. The active fractions (166 ml) were collected.

Step 7. CM-Toyopearl 650M chromatography. The enzyme solution, adjusted to pH 4.0 with 2 M lactic acid, was applied on a column of CM-Toyopearl 650 M (200 ml) (Tosoh Co.). The enzyme was eluted with the buffer supplemented with 400 mM NaCl (pH 4.0). The eluate was adjusted to pH 7.0 with 2 M KOH.

Step 8. Butyl Toyopearl 650 M chromatography. The enzyme solution (50 ml) to which 9.8 g ammonium sulfate was added, was applied on a column of Butyl Toyopearl 650 M (50 ml) (Tosoh Co.). The enzyme was eluted with a linear ammonium sulfate gradient from 1.5 M to 0 M in the buffer.

Step 9. TSK gel DEAE 5PW chromatography. After the enzyme solution dialyzed against the buffer, was concentrated by ultrafiltration to 5 ml, it was applied on a column of TSK gel DEAE 5PW (ϕ 7.5 $\times$ 75 mm) (Tosoh Co.). The enzyme was eluted with a linear NaCl gradient from 90 mM to 150 mM in the buffer.

Step 10. TSK gel Phenyl 5PW chromatography. The enzyme solution (17.8 ml), to which ammonium sulfate was added to 1.5 M, was applied on a column of TSK gel 5PW (ϕ 7.5 $\times$ 75 mm) (Tosoh Co.). The enzyme was

eluted with a linear ammonium sulfate gradient from 1.5 M to 0.75 M in the buffer. The active fraction (26 ml) was collected.

Step 11. TSK gel G3000SW chromatography. The enzyme solution was applied on a column of TSK gel G3000SW (ϕ 7.5 $\times$ 600 mm) (Tosoh Co.). The enzyme was eluted with the buffer supplemented with 1.5 M glycerol at the flow rate of 0.5 ml/min.

Step 12. TSK gel HA-1000 chromatography.

Fig. 1. SDS-Polyacrylamide Gel Electrophoresis.

Lane (A) PUNPI, 2 μ g; Lane (M) phosphorylase B (M_w , 94 k), 3 μ g, bovine serum albumin (M_w , 67 k), 3 μ g, ovalbumin (M_w , 43 k), 3 μ g, carbonic anhydrase (M_w , 67 k), 3 μ g, ovalbumin (M_w , 43 k), 3 μ g, carbonic anhydrase (M_w , 30 k), 3 μ g, soybean trypsin inhibitor (M_w , 20.1 k), 3 μ g, and α -lactalbumin (M_w , 14.4 k), 3 μ g.

Table 1. PURIFICATION OF PURINE NUCLEOSIDE PHOSPHORYLASE

Purification step	Total protein (mg)	Total activity (μ mol/min)	Specific activity (μ mol/min/mg)
1. Dyno mill	22,800	11,200	0.49
2. Heat treatment	18,200	11,100	0.61
3. Acetone treatment	12,800	8,400	0.66
4. Acid treatment	2,800	8,100	2.9
5. 1st DEAE-Toyopearl	550	4,000	7.3
6. 2nd DEAE-Toyopearl	232	3,320	14.3
7. CM-Toyopearl	83.5	3,300	39.5
8. Butyl Toyopearl	22.1	2,940	133
9. TSK gel DEAE 5PW	8.1	2,630	324
10. TSK gel Phenyl 5PW	5.9	1,930	327
11. TSK gel G3000SW	5.7	1,600	280
12. TSK gel HA-1000	1.7	690	405

The enzymatic activity was measured by the method described in the text.

The enzyme solution (4.1 ml) was applied on a column of TSK gel HA-1000 ($\phi 7.5 \times 75$ mm) (Tosoh Co.). The enzyme was eluted with a linear potassium phosphate gradient from 90 mM to 100 mM (pH 6.8). The collected enzyme solution was dialyzed against the buffer and it was used as the enzyme solution.

The enzyme PUNPI was homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1), by isoelectric focusing, and by gel filtration. The results of the purification are summarized in Table I.

Molecular weight

The molecular weight of PUNPI was 68,000 by gel filtration and 34,000 by SDS-polyacrylamide gel electrophoresis (Fig. 1).

Isoelectric point

The isoelectric point of PUNPI was 4.7 by isoelectric focusing (PhastSystem, Pharmacia LKB Biotechnology).

Amino acid composition

The amino acid composition of PUNPI was analyzed. The number of each of the amino acids per subunit was calculated based on the

molecular weight of the subunit (M_w , 34,000) (Table II). No cysteine was found in PUNPI.

Effects of temperature on the reaction

The reaction was done at 40–90°C (Fig. 2). Up to 80°C, hypoxanthine was synthesized with time but heat denaturation was observed at 90°C. When the reaction mixture was heated at 90°C for 1 hr without the enzyme, no hypoxanthine was released.

Fig. 2. Effects of Temperature.

The reaction mixture, containing 0.085 μ g PUNPI in 20 mM inosine and 20 mM potassium phosphate (pH 7.0) in a total volume of 1 ml was incubated at various temperatures. ○, 40°C; ●, 50°C; □, 60°C; ■, 70°C; △, 80°C; ▲, 90°C.

Fig. 3. Effects of pH.

The reaction mixture, containing 0.68 μ g PUNPI in 20 mM inosine and 20 mM potassium phosphate at various pHs in a total volume of 5 ml, was incubated at 60°C for 15 min.

Table II. AMINO ACID COMPOSITION OF PURINE NUCLEOSIDE PHOSPHORYLASE FROM *B. stearothermophilus* JTS859

Amino acid	Residues/Subunit
Asx	22
Thr	11
Ser	8
Glx	34
Gly	27
Ala	29
Val	23
Cys	0
Met	10
Ile	23
Leu	19
Tyr	9
Phe	6
Lys	11
His	5
Arg	16
Pro	15
Try	0

Table III. *K_m* VALUES

The reaction mixture, containing 0.085 μ g PUNPI in 100 mM potassium phosphate and various concentrations of substrate (pH 7.0) in a total volume of 5 ml, was incubated at 60°C.

Substrate	<i>K_m</i> (mM)	Relative <i>V_{max}</i>
Inosine	0.22	100
Guanosine	0.14	133
2'-Deoxyinosine	0.20	100
2'-Deoxyguanosine	0.10	135

Table IV. EFFECTS OF INHIBITORS

The reaction mixture containing 0.09 μ g PUNPI in 1 mM inhibitor, 20 mM inosine, and 20 mM potassium phosphate (pH 7.0) in a volume of 1 ml was incubated at 60°C for 20 min. Liberated hypoxanthine was measured.

Inhibitor	Hypoxanthine (mM) $\pm$ S.D. ^b
Control	0.51 $\pm$ 0.03
PCMB ^a	0
HgCl ₂	0
Iodoacetic acid	0.51 $\pm$ 0.03

^a PCMB, *p*-chloromercuribenzoic acid.

^b S.D., standard deviation (*n* = 3).

Effects of pH on the reaction

A very broad pH-activity profile was obtained as shown in Fig. 3. The optimal pH was from 7.5 to 11.0. When the reaction was done in McIlvaine buffer containing 20 mM potassium phosphate (pH 3.5~7.0) and in 20 mM Tris·HCl buffer containing 20 mM potassium phosphate (pH 7.0~9.5), the pH-activity profile in these buffers were similar to that in potassium phosphate buffer (data not shown).

K_m

K_m values were measured as shown in Table III. PUNPI could not catalyze the phosphorysis of adenosine, 2'-deoxyadenosine, or pyrimidine nucleosides.

Effects of inhibitors on the reaction

The effects of sulfhydryl reagents on PUNPI were examined as shown in Table IV. PUNPI was completely inactivated by PCMB and

Fig. 4. Thermostability of PUNPI

PUNPI (1.7 μ g/ml) was kept in 20 mM potassium phosphate (pH 7.0) (A) and in 20 mM potassium phosphate and 1 mM inosine (pH 7.0) (B) at 70 and 80°C. The remaining activity was measured by the method described in the text. ○, 70°C; ●, 80°C.

HgCl₂ but was not inactivated by iodoacetic acid at a concentration of 1 mM.

Thermostability of PUNPI

As PUNPI was denatured at 90°C as shown in Fig. 2, the thermostability of PUNPI was examined at 70°C and 80°C, and the effects of the substrate, inosine on the thermostability were also examined (Fig. 4). Half-life of PUNPI was 74 min in 20 mM potassium phosphate and 16 hr in 20 mM potassium phosphate and 1 mM inosine at 80°C. Inactivation of PUNPI was not observed at 70°C for the first 30 hr.

Discussion

Thermostable PUNP was purified more than 826-fold from a thermophile, *B. stearothermophilus* JTS859. The PUNPI had an apparent molecular weight of 68,000 with a subunit molecular weight of 34,000, suggesting that the PUNPI consisted of 2 identical subunits. The *K_m* for inosine was 0.22 mM. It was almost the same as that of *B. subtilis* enzymes.⁸⁾

The reported PUNPs have been identified as SH-enzymes.^{9~11)} The effects of pH on the reaction, the effects of sulfhydryl reagents of the reaction, and the amino acid composition

of PUNPI were identified to clarify whether PUNPI was a SH-enzyme or not. The optimal pH range of PUNPI was very broad, pH 7.5~11.0 (Fig. 3). On the other hand the optimal pH range of the reported PUNPs is within 2 pH units including pH 7.0.^{9,12)} The effects of pH on the kinetic properties of the PUNP obtained from rabbit liver and human erythrocytes were investigated in detail.^{9,10)} In both cases, the plots of $\log V_{\max}$ versus pH showed that the enzymatic activity changed at about pH 8.1 and pH 8.5, respectively, suggesting the presence of sulfhydryl groups of cysteine (pK_a 8.3) at the active site of the enzyme. However, the enzymatic activity of PUNPI was almost the same over pHs from 7.5 to 11.0 (Fig. 3). Further, the amino acid analysis showed that PUNPI did not contain cysteine (Table II). These facts suggest that sulfhydryl groups may not be involved in the active site of PUNPI. On the other hand, PUNPI was inhibited by sulfhydryl reagents, as shown in Table IV. As these results were contradictory, further studies are necessary.

The optimal temperature of the reaction was 80°C (Fig. 2). The half-life of the enzyme was 16 hr in 20 mM potassium phosphate and 1 mM inosine (pH 7.0) at 80°C and was 74 min in 20 mM potassium phosphate (pH 7.0) at 80°C (Fig. 4). Stabilization of the enzyme by the presence of the substrate, inosine, was also observed in this case.¹³⁾ The decrease of enzyme activity was not observed at 70°C. The thermostabilities of PUNP obtained from mesophiles at high temperatures have been reported. The residual activity of the PUNP obtained from *Enterobacter cloacae* was 12.5% after 30 min of heating at 70°C in 100 mM

Tris·HCl buffer (pH 7.0).¹⁴⁾ The half-life of the PUNP obtained from *Salmonella typhimurium* was 59 min in 200 mM potassium phosphate and 2 mM 2'-deoxyinosine at 69°C (pH 7.1).¹³⁾ Compared to the thermostability of these PUNPs obtained from mesophiles, PUNPI was extremely stable at high temperature, 80°C. Due to this thermostability, PUNPI is useful for practical applications.

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