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

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Thermostable purine nucleoside phosphorylases, PUNPI and PUNPII, have been purified from *Bacillus stearothermophilus* JTS 859. The characterization of PUNPI was reported previously. [Hori *et al.*, *Agric. Biol. Chem.* 53, 2205 (1989)] PUNPII had a molecular weight of 113,000, consisting of 4 identical subunits (M_w 28,000). The isoelectric point was 5.3. The Michaelis constants for inosine, guanosine, and adenosine were 0.22, 0.34, and 0.075 mM, respectively. The optimal temperature of the reaction was 70°C. The enzyme was stable at 70°C. Although other reported purine nucleoside phosphorylases were SH-enzymes, PUNPII was not a SH-enzyme because the enzyme reaction was not inhibited by PCMB and iodoacetic acid, the optimal pH of the enzyme reaction was from 7.0 to 11.0, and the enzyme did not contain cysteine.

PUNPII and PUNPI were different in several points. Not PUNPI but PUNPII could catalyze the phosphorolysis of adenosine. Specific activity of PUNPI and II for inosine were 405 and 50.6 $\mu\text{mol}/\text{min}/\text{mg}$ protein at 60°C, respectively. PUNPI was stable at 80°C. PUNPII was stable at 70°C, but was denatured at 80°C.

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

purine nucleoside + phosphate

$\rightleftharpoons$ purine base + pentose - 1 - phosphate

Since the substrate specificity of PUNP is not strict, the enzyme has been used to prepare purine nucleoside analogues, such as hypoxanthine arabinoside, virazole, and 6-mercaptopurine nucleoside.^{1~4)}

We have studied the practical application of ribosyl transfer reaction by thermostable PUNP and pyrimidine nucleoside phosphorylase (PYNP) (EC 2.4.2.2) at high temperatures.⁵⁾ We reported the purification and the characterization of a thermostable PUNP, PUNPI from *Bacillus stearothermophilus* JTS 859, and also the existence of another PUNP, PUNPII, in our previous paper.⁶⁾ This paper

describes the purification and the characterization of PUNPII. These two enzymes were different in their substrate specificity, specific activity, and thermal stability. Not PUNPI but PUNPII could catalyze the phosphorolysis of adenosine. The specific activities of PUNPI and II for inosine were 405 and 50.6 $\mu\text{mol}/\text{min}/\text{mg}$ protein at 60°C, respectively. PUNPI was stable at 80°C. PUNPII was stable at 70°C but was denatured at 80°C.

Materials and Methods

Microorganism. *Bacillus stearothermophilus* JTS 859, which produced thermostable purine nucleoside phosphorylases, 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 upper 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 μ l of enzyme solution in a total volume of 1 ml (pH 7.0) was incubated at 60°C for 20 min. Fifty μ l of the reaction mixture was transferred to 950 μ l of MeOH to stop the reaction. The hypoxanthine formed was measured by HPLC.

Analysis. Bases and nucleosides were identified and measured by HPLC. The operating conditions were as follows: column, Chemcosorb 5-ODS-H (ϕ 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 was measured by the method of Lowry *et al.* using bovine serum albumin as a standard.⁷⁾

Measurement of molecular weight. The operating conditions of gel filtration were as follows: 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 (SDS)-polyacrylamide gel electrophoresis was done by the method of Laemmli⁸⁾

Amino acid analysis. The enzyme (16 μ g) was hydrolyzed in 6 N HCl containing 4% thioglycolic acid (400 μ l) at 110°C for 22 hr and 70 hr. Amino acids were analyzed with a Hitachi 835 amino acid analyzer.

Results

Purification

All purification procedures were done at room temperature except dialysis (4°C). Twenty mM potassium phosphate buffer (pH 7.0) used for 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 first supernatant.

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. It was kept at 5 $\sim$ 10°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°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 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 650M (200 ml) (Tosoh Co., Tokyo). The enzyme was eluted with the buffer (2 l) and further with each buffer (1 l) 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, which was reported previously.⁶⁾ Another enzyme eluted with the buffer supplemented with 200 mM NaCl (500 ml), was named PUNPII and its purification and characterization is described in this report.

Step 6. Butyl Toyopearl 650S chromatography. Ammonium sulfate (79.2 g) was added to the enzyme solution followed by adjusting to pH 7.0 with 1 M KOH. This solution was applied on a column of Butyl Toyopearl 650S (100 ml) (Tosoh Co.). The enzyme was eluted with the buffer supplemented with 1.2 M ammonium sulfate (100 ml) (pH 7.0) and further with a linear ammonium sulfate gradient from 1.2 M to 0 M in the buffer (pH 7.0). The active fraction (37.5 ml) was collected.

Step 7. 1st TSK gel G3000SW chromatography. The enzyme solution was applied on a column of TSK gel G3000SW ($\phi 7.5 \times 600$ mm) (Tosoh Co.). The enzyme was eluted with 200 mM potassium phosphate buffer (pH 6.8) at the flow rate at 0.5 ml/min. After dialysis of the collected enzyme solution against the buffer, it was concentrated to 5.8 ml by ultrafiltration.

Step 8. TSK gel DEAE 5PW chromatography. The enzyme solution was applied on a column of TSK gel DEAE 5PW (Tosoh Co.). The enzyme was eluted with a linear NaCl gradient from 0 to 375 mM in 20 mM potassium phosphate buffer (pH 8.0) at a flow rate of 1.0 ml/min.

Step 9. 2nd TSK gel G3000SW chromatography. The collected enzyme was applied on the column of TSK gel G3000SW again. The operation conditions were the same as described in Step 7. The collected enzyme solution was dialyzed against the buffer and it was used as the enzyme solution.

The enzyme, PUNP1I, was shown to be homogeneous by SDS-polyacrylamide gel electrophoresis (Fig. 1), by isoelectric focusing, and by gel filtration. The results of the purification are summarized in Table I.

Molecular weight

Molecular weight of PUNP1I was 113,000 by gel filtration and to be 28,000 by SDS-polyacrylamide gel electrophoresis (Fig. 1).

Fig. 1. SDS-Polyacrylamide Gel Electrophoresis.

Lane (S) PUNP1I, 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 30 k), 3 μ g, soybean trypsin inhibitor (M_w 20.1 k), 3 μ g, and α -lacto albumin (M_w 14.4 k), 3 μ g.

Table I. PURIFICATION OF PURINE NUCLEOSIDE PHOSPHORYLASEII

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. DEAE-Toyopearl	95	139	1.46
6. Butyl Toyopearl	11.3	111.2	9.83
7. 1st TSK gel G3000SW	2.4	69.3	28.9
8. TSK gel DEAE 5PW	0.85	41.3	48.6
9. 2nd TSK gel G3000SW	0.67	34.0	50.6

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

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

Amino acid	Residues/subunit
Asx	18
Thr	18
Ser	9
Glx	25
Gly	20
Ala	25
Val	18
Cys	0
Met	5
Ile	14
Leu	19
Tyr	9
Phe	8
Lys	8
His	4
Arg	13
Pro	4
Try	2

Fig. 2. Effects of Temperature.

The reaction mixture, containing 0.33 μ g PUNP II in 20 mM inosine and 20 mM potassium phosphate (pH 7.0) in a total volume of 1 ml was incubated at various temperatures. $\circ$, 40°C; $\bullet$, 50°C; $\square$, 60°C; $\blacksquare$, 70°C; $\triangle$, 80°C; $\blacktriangle$, 90°C.

Isoelectric point

The isoelectric point of PUNP II was 5.3 by isoelectric focusing (PhastSystem, Pharmacia LKB Biotechnology).

Amino acid composition

The amino acid composition of PUNP II

Fig. 3. Effects of pH in Potassium Phosphate Buffer.

The reaction mixture, containing 0.33 μ g PUNP II in 20 mM inosine and 20 mM potassium phosphate at various pHs in a total volume of 1 ml, was incubated at 60°C for 30 min.

Fig. 4. Effects of pH in Several Buffers.

The reaction mixture containing 0.13 μ g PUNP II, 20 mM inosine and 20 mM potassium phosphate was incubated in the following buffers (20 mM) in a total volume of 0.5 ml at 60°C for 20 min. $\circ$, McIlvaine buffer; $\bullet$, potassium phosphate buffer; $\blacktriangle$, Tris-HCl buffer; $\square$, glycine-NaOH buffer.

was identified. The number of each of the amino acids per subunit was calculated based on the molecular weight of the subunit (28,000) (Table II). Cysteine was not found in PUNP II.

Effects of temperature on the reaction

The reaction was done at 40~90°C (Fig. 2). Hypoxanthine was synthesized with time up to 70°C, but heat denaturation was observed above 80°C. The optimal temperature was estimated to be 70°C. When the reaction mixture was heated at 90°C for 1 hr without the

Table III. *K_m* VALUES

The reaction mixture, containing 0.08 μ g PUNPII in 100 mM potassium phosphate and various concentration of substrate (pH 7.0) in a total volume of 1 ml, was incubated at 60°C.

Substrate	<i>K_m</i> (mM)	Relative <i>V_{max}</i>
Inosine	0.22	100 ^a
Guanosine	0.34	53
Adenosine	0.075	160
2'-Deoxyinosine	0.92	691
2'-Deoxyguanosine	1.9	469
2'-Deoxyadenosine	0.19	172

^a 8.1×10^{-3} μ mol/min/ μ g protein.

enzyme, no hypoxanthine was released.

Effects of pH on the reaction

The effects of pH on the reaction were examined in potassium phosphate buffer and in several other kinds of buffer. Broad pH activity profiles were observed in both cases as shown in Figs. 3 and 4. The optimal pH was from 7.0 to 11.0.

Substrate specificity and *K_m* values

PUNPII could catalyze the phosphorolysis of inosine, guanosine, adenosine, 2'-deoxyinosine, 2'-deoxyguanosine, and 2'-deoxyadenosine but could not catalyze that of xanthosine or pyrimidine nucleosides.

K_m values of these substrates were calculated as shown in Table III.

Effects of inhibitors on the reaction

The effects of sulfhydryl reagents on PUNPII were examined. PUNPII was not inactivated by 1 mM *p*-chloromercuribenzoate (PCMB) or iodoacetic acid as shown in Table IV. These results showed that sulfhydryl groups were not involved in the active site of PUNPII.

Thermostability of PUNPII

Thermostability of PUNPII was examined at 70°C and 80°C, and the effects of the substrate (inosine) on the thermostability were also examined (Fig. 5). PUNPII was stable in

Table IV. EFFECTS OF INHIBITORS

The reaction mixture containing 0.33 μ g PUNPII in 1 mM each 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 formed (mM) $\pm$ SD ^b
Control	0.39 ± 0.04
PCMB ^a	0.39 ± 0.03
Iodoacetic acid	0.42 ± 0.01

^a PCMB, *p*-chloromercuribenzoate.

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

Fig. 5. Thermostability of PUNPII.

PUNPII (8.9 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. $\circ$, 70°C; $\bullet$, 80°C.

20 mM potassium phosphate at 70°C, although a slight decrease in activity was observed, while the residual activity of PUNPII was 38% in the presence of inosine after 3 hr of incubation at 70°C. The half-life of PUNPII was 15 min in 20 mM potassium phosphate and the residual activity was 13% after 1 hr of incubation in 20 mM potassium phosphate at 80°C. On the other hand, the residual activity was 4% after 1 hr of incubation in 20 mM potassium phosphate and 1 mM inosine (pH 7.0) at 80°C. These results showed that PUNPII was not stabilized by inosine.

Discussion

It was found that *B. stearothermophilus* JTS

859 produced two different PUNPs, PUNPI⁽⁶⁾ and PUNPII. PUNPII had an apparent molecular weight of 113,000 with a subunit molecular weight of 28,000, suggesting that PUNPII consisted of four identical subunits. On the other hand, the molecular weight of PUNPI was 68,000 and it consisted of two identical subunits (M_w 34,000).⁽⁶⁾

PUNPII could catalyze the phosphorolysis of purine nucleosides including adenosine and 2'-deoxyadenosine, while PUNPI could not catalyze that of adenosine and 2'-deoxyadenosine. *Km* values of PUNPII for inosine and guanosine were similar to those of PUNPI. On the other hand, the amount and the specific activity of PUNPI were 29 times larger and 8 times higher than those of PUNPII, respectively. Therefore, it is supposed that PUNPI catalyze the phosphorolysis of inosine and guanosine and PUNPII mainly catalyze that of adenosine in the nucleoside salvage pathway. It has been reported that two different PUNPs had different functions in the nucleoside salvage pathway in *B. subtilis*.⁽⁹⁾

PUNPII was stable at 70°C but was denatured at 80°C (Figs. 2, 5). The half-life of PUNPI at 80°C was 16 hr, but PUNPII was almost inactivated after only 1 hr in 20 mM potassium phosphate and 1 mM inosine (pH 7.0). Further, stabilization of PUNPII in the presence of substrate (inosine) was not observed, although this kind of stabilization were observed with PUNPI and other PUNPs^(10,11). It is interesting that these two PUNPs, I and II were very different in thermostability.

The PUNPs reported so far have been identified as SH-enzymes based on the inhibition by PCMB and the effects of pH on the reaction rate.^(12~14) However, it was concluded that PUNPII was not SH-enzyme because PUNPII

was not inactivated by PCMB or iodoacetic acid (Table IV), the optimal pH of the reaction was very broad, pH 7.0~11.0 (Figs. 3,4), the enzymatic activity was not changed at about pH 8.3 (cysteine SH pK_a = 8.3), suggesting that sulfhydryl groups were not involved in the active site of PUNPII, and the amino acid analysis showed that PUNPII did not contain cysteine (Table II). PUNPII might be advantageous for practical applications due to the broad optimal pH range based on the non SH-enzyme.

References

- 1) T. Utagawa, H. Morisawa, S. Yamanaka, A. Yamazaki, F. Yoshinaga and Y. Hirose, *Agric. Biol. Chem.*, **49**, 2425 (1985).
- 2) T. Utagawa, H. Morisawa, S. Yamanaka, A. Yamazaki, F. Yoshinaga and Y. Hirose, *Agric. Biol. Chem.*, **50**, 121 (1986).
- 3) R. Gardner and A. Kornberg, *J. Biol. Chem.*, **242**, 2383 (1967).
- 4) J. D. Stockler, C. Cambor and R. E. Parks, Jr., *Biochemistry*, **19**, 102 (1980).
- 5) N. Hori, M. Watanabe, Y. Yamazaki and Y. Mikami, *Agric. Biol. Chem.*, **53**, 197 (1989).
- 6) N. Hori, M. Watanabe, Y. Yamazaki and Y. Mikami, *Agric. Biol. Chem.*, **53**, 2205 (1989).
- 7) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 8) U. K. Laemmli, *Nature*, **227**, 680 (1970).
- 9) K. Jensen, *Biochim. Biophys. Acta*, **525**, 346 (1978).
- 10) K. F. Jensen and P. Nygaard, *Eur. J. Biochem.*, **51**, 253 (1975).
- 11) T. A. Krentisky, G. W. Koszalka and J. V. Tuttle, *Biochemistry*, **20**, 3615 (1981).
- 12) A. S. Lewis and M. D. Glantz, *J. Biol. Chem.*, **251**, 407 (1976).
- 13) A. S. Lewis and M. D. Glantz, *Biochemistry*, **15**, 4451 (1976).
- 14) R. P. Agarwal and R. E. Parks, Jr., *J. Biol. Chem.*, **244**, 644 (1969).