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Purification and Characterization of a Novel Nucleoside Phosphorylase from a *Klebsiella* sp. and Its Use in the Enzymatic Production of Adenine Arabinoside

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An adenosine-assimilating bacterium, *Klebsiella* sp. strain LF1202, inducibly formed a novel nucleoside phosphorylase which acted on both purine and pyrimidine nucleosides when the cells were cultured in medium containing adenosine as a sole source of carbon and nitrogen. The enzyme was purified (approximately 83-fold, with a 17% activity yield) to the homogeneous state by polyacrylamide gel electrophoresis. The molecular weight of the purified enzyme was calculated to be 125,000 by gel filtration of Sephadex G-200 column chromatography, although the enzyme migrated as a single protein band with a molecular weight of 25,000 on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis; thus, it was thought to consist of five identical subunits. Besides purine nucleosides (adenosine, inosine, and guanosine), the purified enzyme also acted on pyrimidine nucleosides such as uridine, 2′-deoxyuridine, and thymidine. The purified enzyme catalyzed the synthesis of adenine arabinoside, a selective antiviral pharmaceutic agent, from uridine arabinoside and adenine.

Purine and pyrimidine salvage systems in bacteria have been well characterized in *Escherichia coli* and *Salmonella typhimurium* (11). Purine or pyrimidine nucleosides are specifically phosphorolyzed to pentose-1-phosphate and either purine or pyrimidine bases, respectively, in the presence of P_i. The reactions are catalyzed by purine nucleoside phosphorylase (PNPase; EC 2.4.2.1) or pyrimidine nucleoside phosphorylase (PyNPase; EC 2.4.2.2), i.e., uridine phosphorylase (UPase; EC 2.4.2.3) and thymidine phosphorylase (TPase; EC 2.4.2.4). Cytidine is deaminated to uridine by an action of cytidine deaminase (EC 3.5.4.5), and then the uridine is degraded to uracil and ribose-1-phosphate by UPase. PNPase or PyNPase also catalyze the phosphate-dependent pentosyl moiety transfer between purine or pyrimidine base and purine or pyrimidine nucleoside to yield another purine or pyrimidine nucleoside.

Recently, the utilization of PNPase for the enzymatic production of purine nucleoside analogs, which are approved for antiviral pharmaceutic drugs such as adenine arabinoside (AraA), has been developed (13). Previously, we screened bacteria which are able to utilize adenosine as a sole source of carbon and nitrogen from soil in order to obtain a potent producer of PNPase (F. Ling, Y. Inoue, and A. Kimura, Agric. Biol. Chem., in press). A bacterium which can utilize adenosine as a sole source of carbon and nitrogen would be expected to have a strong PNPase activity in the cells. A bacterium obtained was identified to be a *Klebsiella* sp. and was designated strain LF1202 (Ling et al., in press).

In this article, we describe the purification and characterization of a novel nucleoside phosphorylase (NPase) which acts on both purine and pyrimidine nucleosides and also demonstrate the enzymatic production of AraA from uridine arabinoside (AraU) and adenine by using the enzyme.

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MATERIALS AND METHODS

Chemicals. All purine and pyrimidine nucleosides and xanthine oxidase were purchased from Sigma Chemical Co., St. Louis, Mo. Other chemicals were all analytical grade reagents.

Microorganisms and cultures. *Klebsiella* sp. strain LF1202, isolated and identified previously (Ling et al., in press), was cultured in AMT medium (0.5% adenosine, 0.01% $MgCl_2$, 0.1% sodium tetrapolyphosphate [pH 7.2]) at 30°C with reciprocal shaking (120 rpm) for 20 h.

Enzyme assay. The standard reaction mixture (1.0 ml) contained 5.0 mM inosine, 10 mM NaH_2PO_4, 0.05 U of xanthine oxidase per ml, 100 mM Tris-HCl buffer (pH 8.0), and enzyme. The reaction was started by the addition of the enzyme at 25°C, and increase of the A_{290} was measured. One unit of activity was defined as the amount of enzyme forming a micromole of uric acid per minute by using a value of 12.0 as the millimolar extinction coefficient. Protein was determined by the method of Lowry et al. (10).

Purification of NPase. (i) Cell extracts. The cells (12 g [wet weight]), obtained from a 10-liter culture of AMT medium, were washed once with a 0.85% NaCl solution, resuspended in 110 ml of 10 mM Tris-HCl buffer (pH 7.0), and disrupted by KUBOTA Insonator Model 200M at 90 kHz. The homogenates were centrifuged at 25,000 × *g* for 30 min, and resultant supernatants were dialyzed against the same buffer. All purification procedures were carried out at 0 to 4°C.

(ii) DEAE-cellulose column chromatography. The dialysate (120 ml, 857.7 mg of protein) was applied to a DEAE-cellulose column (4.5 by 20 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.0), and the proteins were eluted with a linear gradient of KCl (0 to 1.0 M; total volume, 1,000 ml) in the same buffer. Fractions were collected as 5-ml portions per tube. The active fractions (conductivity, 2.5 to 4.0 mS) were pooled (85 ml, 130.7 mg of protein).

(iii) Butyl Toyopearl 650M column chromatography. Solid ammonium sulfate (15 g, 30% saturation) was added to the

TABLE 1. Purification of NPase from *Klebsiella* sp. strain LF1202

Step	Protein (mg)	Total activity (U)	Sp act (U/mg of protein)	Yield (%)	Purification (fold)[a]
Crude extract	857.7	448.8	0.52	100	1.0
DEAE-cellulose	130.7	331.1	2.54	74.4	4.8
Butyl-Toyopearl 650M	9.4	308.5	32.8	69.4	63.4
Sephadex G-200	1.8	75.9	42.8	17.1	82.7

[a] Values are given relative to the specific activity of crude extract.

active fractions obtained as described above, and the mixture was kept for 2 h. The precipitates without activity were removed after centrifugation at 15,000 rpm for 20 min. The resultant supernatants were applied onto a Butyl Toyopearl 650M column (4.5 by 7.0 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.0) containing 30% ammonium sulfate. The proteins adsorbed were eluted with a decreasing gradient of ammonium sulfate (30 to 0%; total volume, 600 ml) in the same buffer. Fractions were collected as 5-ml portions per tube. The active fractions (conductivity, 9.0 to 8.5 mS) were collected and concentrated to 3 ml by an Amicon PM10 membrane.

(iv) Sephadex G-200 column chromatography. The concentrate (3 ml, 1.8 mg of protein) was loaded onto a Sephadex G-200 column (1.5 by 100 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.0). Fractions were collected as 1-ml portions per tube, and the active fractions (no. 82 to 90) were combined and used for the characterization of the enzyme.

Molecular weight determination. The molecular weight of the purified enzyme was determined by gel filtration with a Sephadex G-200 column (1.5 by 100 cm) according to the method of Andrews (2).

PAGE. Polyacrylamide (12.5%) gel electrophoresis (PAGE) in the presence or absence of 0.1% sodium dodecyl sulfate (SDS) was conducted according to the method of Laemmli (8). The gels were stained for proteins with Coomassie brilliant blue.

Enzymatic production of AraA. The synthesis of AraA was carried out in a mixture (1.0 ml) containing 1.0 mM adenine, 3.0 mM AraU, 100 mM potassium phosphate buffer (pH 8.0), and 0.86 U of purified enzyme per ml at 50°C for 20 h. An aliquot (20 μl) of the reaction mixture was withdrawn and analyzed by paper chromatography (solvent system, isobutyric acid–0.5 M ammonia [5:3, vol/vol]) with TOYO filter paper 51B. The spot corresponding to AraA was cut off under UV illumination (254 nm) and extracted with 0.01 N HCl at 25°C for 16 h. The identity of this spot has been previously confirmed by Yokozeki et al. (14). The concentration of AraA was determined by using a value of 12.6 as the millimolar extinction coefficient at 257 nm.

RESULTS

Purification and properties of NPase. The overall purification procedures for NPase are summarized in Table 1. The enzyme was purified approximately 83-fold with a 17% activity yield. Purity of the enzyme at various purification steps was analyzed by PAGE (Fig. 1A), and the active peaks after Sephadex G-200 column chromatography gave a single protein band (Fig. 1A, lane 4). The molecular weight of the purified enzyme was estimated to be 125,000 on a calibration column of Sephadex G-200. The enzyme treated with dithiothreitol migrated as a single protein band with a molecular

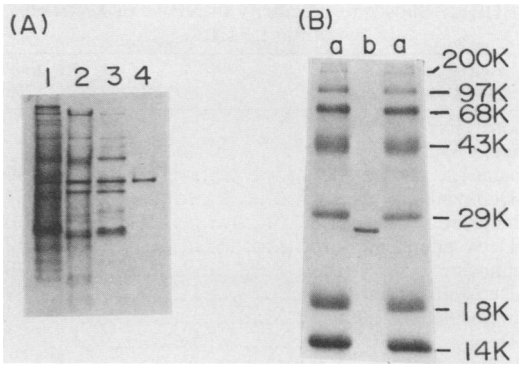

FIG. 1. PAGE of NPase. (A) Samples at various purification steps were analyzed by native PAGE. The samples and the amount of proteins (in μg) were as follows: lane 1, cell extracts (200); lane 2, after DEAE-cellulose column (100); lane 3, after Butyl-Toyopearl 650M column (85); lane 4, after Sephadex G-200 column (45). (B) The purified enzyme (30 μg) was treated with SDS in the presence of dithiothreitol and analyzed by SDS-PAGE. Lanes a, molecular weight markers, from top to bottom: myosin (H chain) (M_r, 200,000), phosphorylase *b* (M_r, 97,400), bovine serum albumin (M_r, 68,000), ovalbumin (M_r, 43,000), carbonic anhydrase (M_r, 29,000), β-lactoglobulin (M_r, 18,400), and lysozyme (M_r, 14,300). Lane b, NPase.

weight of 25,000 on SDS-PAGE (Fig. 1B, lane b). Thus, the enzyme was suggested to consist of five identical subunits. The optimal activity of the enzyme for phosphorolysis and synthesis of inosine and uridine was obtained at 40°C and a pH of 8.0. The enzyme was stable up to 60°C and more than 85% of the enzyme activity remained even after incubation of the enzyme at 50°C for 24 h. Effects of various compounds and bivalent metal ions on the activity were investigated. Among the chemicals tested, neither activator nor inhibitor was found (data not shown).

Kinetics. The reaction proceeded by a Michaelis-Menten type mechanism. The K_m values for inosine and P_i in the forward reaction (phosphorolysis of nucleoside) were calculated to be 0.66 and 0.56 mM, respectively. The K_m values for hypoxanthine and ribose-1-phosphate in the reverse reaction (synthesis of purine nucleoside) were estimated to be 0.45 μM and 0.14 mM, respectively. On the other hand, K_m values for uridine in forward and uracil in reverse reactions were 0.38 and 0.44 mM, respectively.

Substrate specificity. The purified enzyme catalyzed both phosphorolysis and synthesis of purine nucleosides, except for xanthosine (Table 2). In the forward (phosphorolysis of purine nucleoside) reaction, 2′-deoxyinosine was phosphorolyzed to a 2.5-fold greater extent than inosine. Neither xanthosine nor 2′-deoxyxanthosine served as the substrate for the purified enzyme. In addition to purine nucleosides, the enzyme was also active toward pyrimidine nucleosides, such as uridine, 2′-deoxyuridine, and thymidine. The enzyme was most active on uridine, and the relative activity for uridine was threefold higher than that for inosine, but the activity for thymidine was only one-third of that of inosine. The enzyme also catalyzed the synthesis of pyrimidine nucleosides from pyrimidine bases, i.e., uracil and thymine, and ribose- and/or 2′-deoxyribose-1-phosphate. The substrate specificity of the purified enzyme was different from that of other PNPases or PyNPases, and the enzyme should therefore be designated NPase rather than PNPase or PyNPase.

Enzymatic production of AraA. The optimal conditions for

TABLE 2. Substrate specificity of NPase of *Klebsiella* sp. strain LF1202

Activity and substrate[a]		Relative activity (%)[b]
Phosphorolysis		
Purines		
Inosine		100
2'-Deoxyinosine		254
Adenosine		108
2'-Deoxyadenosine		101
Guanosine		100
2'-Deoxyguanosine		98
Xanthosine		0
2'-Deoxyxanthosine		0
Pyrimidines		
Uridine		368
2'-Deoxyuridine		95
Cytidine		0
2'-Deoxycytidine		0
Thymidine		29
Synthesis		
Purines		
Hypoxanthine	Rib-1-P	100
	dRib-1-P	104
Adenine	Rib-1-P	137
	dRib-1-P	127
Guanine	Rib-1-P	77
	dRib-1-P	63
Xanthine	Rib-1-P	0
	dRib-1-P	0
Pyrimidines		
Uracil	Rib-1-P	82
	dRib-1-P	39
Cytosine	Rib-1-P	0
	dRib-1-P	0
Thymine	dRib-1-P	17

[a] Rib-1-P, Ribose-1-phosphate; dRib-1-P, 2'-deoxyribose-1-phosphate.
[b] Phosphorolysis of inosine is given as 100%; other phosphorolysis activities are given relative to that value. Synthesis of hypoxanthine ribose-1-phosphate is given as 100%; other synthesis activities are given relative to that value.

the enzymatic production of AraA from AraU and adenine with NPase were investigated (Fig. 2). pH, temperature, concentration of P_i, and the ratio of AraU to adenine were examined (Fig. 3). The maximum production of AraA was obtained in a reaction mixture containing 1.0 mM adenine, 3.0 mM AraU, 100 mM potassium phosphate buffer (pH 8.0),

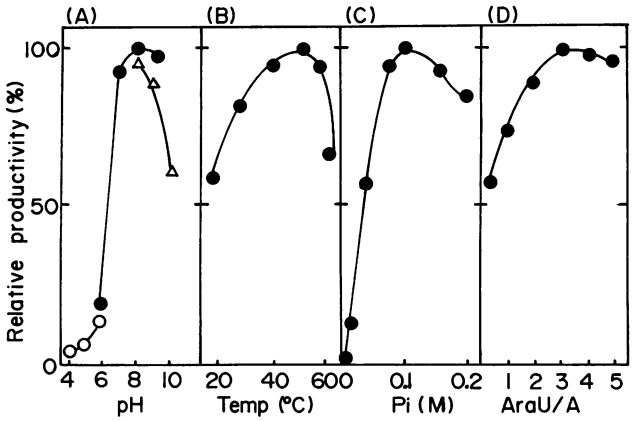

FIG. 3. Effects of pH (A), temperature (B), concentration of P_i (C), and the ratio of AraU to adenine (D) on the production of AraA. (A) The buffers used were sodium acetate buffer (pH 4 to 6) (○), potassium phosphate buffer (pH 6 to 9) (●), and Tris-HCl buffer (pH 7 to 10) (△). The relative productivity at pH 8.0 in potassium phosphate buffer was taken as 100%. (B) The relative productivity at 50°C was taken as 100%. (C) The concentration of potassium phosphate buffer (pH 8.0) in the reaction mixture was varied as indicated, and the relative productivity at 0.1 M was taken as 100%. (D) The concentration of adenine (A) was fixed at 1 mM, and the concentration of AraU was varied as indicated. The relative productivity at AraU/A = 3 was taken as 100%.

and 0.86 U of NPase per ml at 50°C. Fig. 4 shows the time course of the enzymatic production of AraA under the optimal conditions described above. The concentration of adenine and AraU in the reaction mixture, in which the ratio of AraU to adenine was fixed at 3, was varied as indicated in Fig. 4. AraA in the reaction mixture increased in association with the incubation time, and approximately 30% of AraU was converted to AraA after a 30-h incubation. At this time, adenine was added to the reaction mixture to bring the ratio of residual AraU to adenine to 3, and the mixture was then incubated for another 20 h. AraA in the reaction mixture increased after the addition of adenine, and 40 to 45% of AraU was converted to AraA.

DISCUSSION

Since an enzyme found in *Klebsiella* sp. strain LF1202 has both PNPase and PyNPase activities, it was designated

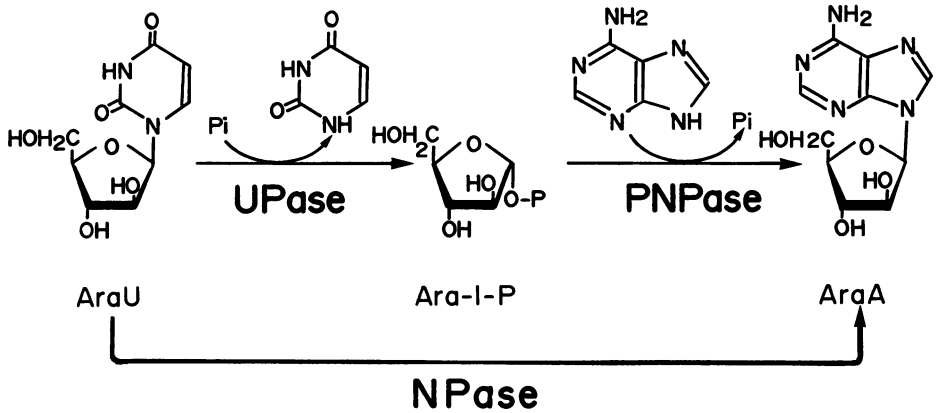

FIG. 2. Scheme of enzymatic production of AraA by NPase. Ara-1-P, Arabinose-1-phosphate.

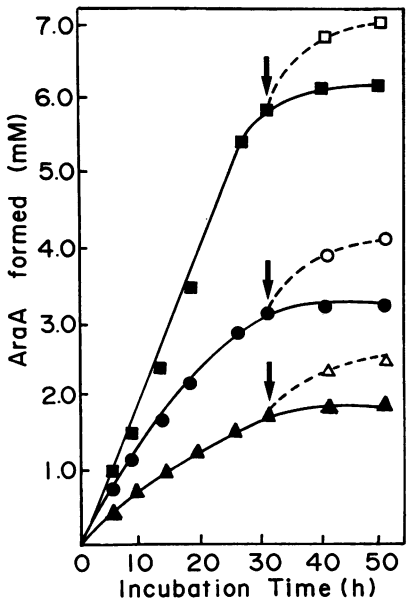

FIG. 4. Enzymatic production of AraA by NPase. The reaction was carried out under the optimal conditions as described in the legend to Fig. 3. Concentrations of adenine at the start of the reaction were 2 (▲), 3.3 (●), and 6.7 mM (■). The arrows indicate the time when additional adenine was added.

NPase. The molecular weight of the purified NPase was calculated to be 125,000 on gel filtration of Sephadex G-200 column chromatography, whereas the purified enzyme migrated as a single protein band with a molecular weight of 25,000 on SDS-PAGE (Fig. 1B). Thus, the enzyme was suggested to consist of five identical subunits. In *Escherichia coli*, the occurrence of two kinds of PNPases, the *deoD* gene product and the *xapA* gene product, has been reported (3, 5, 6, 11). The molecular weight of the *deoD*-encoded PNPase was 138,000 ± 10%, and the enzyme consisted of six identical subunits with molecular weights of 23,700 ± 5% (6). The latter gene (*xapA*) is expressed only when the cells are grown in a medium containing xanthosine and so was designated xanthosine phosphorylase (3, 5). Recently, Koszalka et al. have partially purified *xapA*-encoded PNPase from *E. coli* cells lacking the *deo* operon and estimated the molecular weight of this protein to be 180,000, although the subunit structure has not been extensively studied (7). PNPases have been also purified from human erythrocytes (1) and rat liver (9). The molecular weight of the former enzyme was 80,000 and its subunit structure has not been studied, whereas the latter consisted of a single polypeptide chain with a molecular weight of 39,000 to 40,000.

The *deoD*-encoded PNPase of *E. coli* was inactivated by *p*-chloromercuribenzoate (PCMB), and the enzyme was dissociated into subunits (6). On the other hand, *xapA*-encoded PNPase was not inactivated by PCMB (7). Inactivation and dissociation of the subunits of PNPase by PCMB were also observed in *Salmonella typhimurium* enzyme (6). NPase purified from *Klebsiella* sp. strain LF1202 was neither inactivated nor dissociated by PCMB; thus, the enzyme was suggested to contain no active-site thiol.

Besides having nucleoside phosphorolysis activity, the enzyme purified from *Klebsiella* sp. strain LF1202 catalyzed the phosphate-dependent pentosyl moiety-exchanging reaction between AraU and adenine. By the use of the novel characteristic of the purified enzyme, the enzymatic production of AraA was demonstrated. Utagawa and his colleagues reported excellent studies on the enzymatic production of AraA by the use of cell extracts of *Enterobacter aerogenes* in which two kinds of enzymes (UPase and PNPase) were included (Fig. 2) (13). However, the optimal conditions of these two enzymes are different (12); thus, there remained several problems in obtaining a high yield. We adapted a novel NPase to an enzymatic production of AraA from AraU and adenine and established the optimal condition for one-step production. Approximately 40 to 45% of AraU was converted to AraA at a relatively low temperature (50°C) compared with that reported by Utagawa et al. (60°C) (13). As observed by Utagawa et al. (13), the reaction for synthesis of AraA proceeded slowly. Since NPase was active on both purine and pyrimidine nucleosides, AraA being synthesized from AraU and adenine may be phosphorolyzed to adenine and arabinose-1-phosphate. To obtain high productivity, AraA should be withdrawn from the reaction system. We are now trying to construct a method for elimination of AraA from the reaction mixture. We are also searching for chemicals which increase the production of AraA.

The NPase reported in this article has a novel characteristic; the enzyme would be expected to be a good model for the study of the catalytic mechanism in protein engineering. In order to obtain a large amount of NPase, molecular cloning of the gene corresponding to the NPase from *Klebsiella* sp. strain LF1202 is now in progress.

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