Purification and Characterization of Ascorbic Acid Phosphorylating Enzyme from *Pseudomonas azotocolligans*

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A enzyme that catalyzed the specific formation of ascorbic acid-2-phosphate (AsA2P) from ascorbic acid (AsA) and adenosine-5'-triphosphate (ATP), was purified 3,200-fold to homogeneity from a cell extract of *Pseudomonas azotocolligans*. The purified enzyme appeared as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and consisted of a single polypeptide with a molecular weight of about 30,000. Of phosphoryl donors tested, *p*-nitrophenylphosphate (*p*-NPP) and pyrophosphate (PPi) were as effective as ATP. Optimal pHs for the phosphorylating activity were around 4.0 and 5.5 when PPi and ATP were used as phosphoryl donors, respectively. The *K_m* for AsA was 147 mM. The enzyme activity was inhibited by Cu²⁺, but not by sulfhydryl reagents.

The enzyme simultaneously had phosphatase activity at weakly acidic or neutral pH and the *K_m* for *p*-NPP in the phosphatase activity was 0.38 mM. The enzyme was tentatively named "ascorbic acid phosphorylating enzyme."

Ascorbic acid (AsA) is widely used, for example, in medicines, foodstuffs, and cosmetics, but has the disadvantage that it is susceptible to thermal and oxidative degradation. Modifications of AsA to a more stable form have been reported from a number of laboratories.^{1–6)} Among them were ascorbic acid-2-phosphate (AsA2P),⁷⁾ ascorbic acid-2-sulfate, and ascorbic acid α -glucoside.⁸⁾ AsA2P shows very high stability *in vitro*, and is easily converted to AsA *in vivo* by enzymatic dephosphorylation (Fig. 1). AsA2P has been shown to promote fibroblast growth and have

an antiscorbutic activity like AsA.⁹⁾

In our previous paper,¹⁰⁾ we described the existence of the enzyme activity in *Pseudomonas azotocolligans* KY4661 that catalyzed the specific formation of AsA2P from AsA and ATP, a novel reaction not previously reported.

Here we report the purification and characterization of the enzyme named "ascorbic acid phosphorylating enzyme (APE)" from *P. azotocolligans* KY4661.

Materials and Methods

Chemicals. Ascorbic acid-2-phosphate (AsA2P) was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. The standard marker proteins for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and high performance liquid chromatography (HPLC) were from Bio-Rad Laboratories and Pharmacia LKB Biotechnology Inc, respectively. All other chemicals used were commercially available and of analytical grade.

Microorganism and growth conditions. *P. azotocolligans* KY4661 was from type cultures preserved in our

Fig. 1. The Structure of AsA and AsA2P.

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laboratory. Cells were grown in a test tube containing 10 ml of nutrient medium composed of 1% glucose, 1% polypeptone, 0.7% meat extract, 0.5% yeast extract, and 0.3% NaCl (pH 7.2), with reciprocal shaking at 28°C for 24 hr. The cells were transferred into a 2-liter shaking flask containing 140 ml of the same medium and grown at 28°C for 24 hr with rotary shaking. The resultant subculture was inoculated into a 5-l jar fermentor (type KMJ-5-6; Mitsuwa Co., Japan) containing 2.5 l of the same fresh medium. Cultivation was done at 28°C for 24 hr with aeration (1 l per liter of medium per min) and agitation (600 rpm). The grown cells were harvested by centrifugation at 4°C.

Assay for AsA2P formation. The standard reaction mixture (1.0 ml) consisted of 40 mM potassium phosphate buffer (PKB) (pH 5.5), 200 mM AsA, 40 mM ATP, 10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and enzyme. The reaction was done at 30°C for 150 min, and the AsA2P formed was measured by HPLC. One unit of APE activity was defined as the amount of enzyme that produced 1 μmol of AsA2P per min.

Phosphatase assay. The reaction mixture contained 0.1 M different kinds of buffers, enzyme, and 5 mM *p*-NPP or 62 mM AsA2P. The *p*-nitrophenol (*p*-NP) formed was measured at 400 nm under alkaline conditions then calculated from the standard curve obtained from authentic *p*-NP. AsA formed was measured by HPLC.

Analysis. AsA and AsA2P was measured by HPLC as described previously.¹⁰⁾ Protein was measured by a Bio-Rad protein assay kit with γ -globulin as the standard.

Purification of APE from *P. azotocolligans*. All purification procedures were done at 0 to 4°C.

Step 1. Preparation of cell extracts. Cells (570 g, wet weight) were suspended in 5.7 l of 0.01 M PKB (pH 7.0) and then disrupted with a Manton-Gaulin Laboratory Homogenizer (Manton-Gaulin Manufacturing Co., Inc., U.S.A.). The cell debris was removed by centrifugation (10,000 $\times g$ for 30 min).

Step 2. Ammonium sulfate fractionation. The cell extracts were fractionated by salting out with solid ammonium sulfate to 30 to 50% saturation. The precipitate formed was collected by centrifugation at 10,000 $\times g$ for 30 min, dissolved in 1 l of 0.01 M PKB (pH 7.0), and then dialyzed against the same buffer.

Step 3. CM-Sephadex column chromatography. The dialyzed fraction was adjusted to pH 6.0 with phosphoric acid, and then put on a CM-Sephadex column (7.4 $\times$ 19 cm), previously equilibrated with 0.02 M PKB (pH 6.0). After washing the column with the same buffer, the enzyme was eluted with a linear gradient of 0 to 0.25 M NaCl in the buffer. Active fractions were combined and concentrated to 44 ml with an Amicon YM10 membrane.

Step 4. Chromatofocusing. The concentrated enzyme fraction was put on a Sephadex G-25 column (2.2 $\times$ 39 cm), previously equilibrated with 0.025 M triethylamine-HCl

(pH 11.0). Protein was desalted by elution with 0.025 M triethylamine-HCl (pH 11.0). The void peak containing material absorbing at A_{280} was pooled and concentrated to 28 ml with an Amicon YM10 membrane. The desalted protein was put on a PBE118 column (1.4 $\times$ 55 cm) equilibrated with 0.025 M triethylamine-HCl (pH 11.0). Enzyme was eluted with Pharmalyte pH 8–10.5-HCl (pH 7.5). The active fractions were pooled and concentrated as before.

Step 5. Sephadex G-75 chromatography. The concentrated protein fraction was put on a Sephadex G-75 column (2.3 $\times$ 80 cm), previously equilibrated with 0.02 M PKB (pH 7.0). The protein was eluted with the same buffer and the active fractions were pooled, concentrated as before, and stored at 0°C until use.

Amino acid analysis. A sample of APE (1.2 mg) was hydrolyzed in 6 N HCl and the amino acid was analyzed with a JOEL JLC-300 amino acid analyzer.

Measurement of molecular weight. Estimation of the molecular weight of the enzyme by gel filtration was done on a Shim-pack DIOL-150 column (7.9 mm $\times$ 50 cm, Shimadzu Seisakusho, Tokyo, Japan) eluted with 0.1 M potassium phosphate buffer containing 0.2 M NaCl at a flow rate of 1.0 ml per min.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was done by the method of Laemmli.¹¹⁾ Proteins were stained with Coomassie brilliant blue R-250.

Results

Purification of APE

A typical purification procedure of *P. azotocolligans* APE is summarized in Table I. The enzyme was purified approximately 3,200-fold from the cell extract with a yield of 16%. The preparation at the final step of purification had a single band on SDS-PAGE (Fig. 2).

Molecular weight

The molecular weight of the enzyme was estimated to be about 31,500 by gel filtration, and 29,000 by SDS-PAGE (Fig. 2). Thus, the enzyme appears to be a monomeric structure.

Amino acid composition

The amino acid composition of the purified enzyme is given in Table II. The number of phenylalanine residues per mole could not be

Table I. PURIFICATION OF APE FROM *P. azotocolligans*

Step	Protein (mg)	Total activity (U ^a)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Cell extract	102,620	210	0.002	100	1
Ammonium sulfate	25,534	190	0.008	91	4
CM-Sepharose	977	170	0.173	81	85
Chromatofocusing	12.7	38	2.90	18	1417
Sephadex G-75	5.1	33	6.39	16	3196

^a 1U = 1 μ mol AsA2P per min.

Table II. AMINO ACID COMPOSITION OF APE

Amino acid	Mol%	Residues/mol ^a
Asx	8.74	22
Thr	5.94	15
Ser	4.95	13
Glx	8.24	21
Pro	8.41	22
Gly	8.41	22
Ala	20.94	54
Val	4.12	11
Met	2.64	7
Ile	1.56	4
Leu	7.17	18
Tyr	2.89	7
Phe	N.D.	N.D.
Lys	2.72	7
His	3.22	8
Arg	9.32	24
Trp	0.66	2
Cys ^b	—	—

N.D. = not detected.

^a Nearest integral number calculated on the basis of the molecular weight of 31,500 estimated by Shim-pack DIOL-150 gel filtration.

^b Not determined.

Table III. PHOSPHORYL DONOR SPECIFICITY OF APE

Substrate	pH	Relative activity
ATP	5.6	100
ATP	4.3	22
Acetylphosphate	4.1	145
<i>p</i> -NPP	4.1	111
Pyrophosphate	4.1	114
Triphosphate	4.1	83
Tetraphosphate	4.1	76
Phosphoric acid	4.1	0

calculated to be 24 mM and 10 mM, respectively. Isoascorbic acid, a stereoisomer of L-ascorbic acid, was also phosphorylated by the purified

Fig. 2. Molecular Weight Measurement.

The purified enzyme was treated with SDS in the presence of 2-mercaptoethanol and electrophoresed on a 0.1% SDS–12.5% polyacrylamide gel. Lane a, APE; b, molecular weight markers: phosphorylase *B* (M_r 92,500); bovine serum albumin (M_r 66,200); ovalbumin (M_r 45,000); carbonic anhydrase (M_r 31,000); soybean trypsin inhibitor (M_r 21,500); lysozyme (M_r 14,400).

accurately measured because of the low contents in the protein.

Substrate specificity

The activity of the enzyme to form AsA2P from AsA and various phosphoryl donors are given in Table III. Besides ATP, acetylphosphate, *p*-NPP, PPi, triphosphate, and tetraphosphate were also found to be used as phosphoryl donors, but phosphoric acid was inert. The apparent K_m for ATP and PPi were

Fig. 3. Effects of pH of APE Activity.

The activity of the enzyme was measured in the standard assay conditions described in Materials and Methods, except for buffer. The buffers used were (○, △) sodium acetate buffer and (●, ▲) potassium phosphate buffer. The substrate were (△, ▲) ATP and (○, ●) PPI. The activity at pH 5.5 for ATP and at 4.0 for PPI was taken as 100%.

Table IV. EFFECTS OF VARIOUS CHEMICALS ON APE ACTIVITY

The reaction mixture contained 40 mM acetate buffer, 200 mM AsA, 100 mM PPI, and enzyme. The enzyme was incubated with a compound for 10 min before the reaction was started with AsA and PPI.

Compound added	Concentration (mM)	Relative activity
None	—	100
Ca ²⁺	1.0	109
Mg ²⁺	1.0	76
Mg ²⁺	10.0	121
Fe ²⁺	1.0	121
Mn ²⁺	1.0	112
Cu ²⁺	1.0	64
Cu ²⁺	10.0	32
NaF	10.0	112
EDTA	10.0	112
NaN ₃	1.0	87
α,α'-Dipyridyl	1.0	86
8-Hydroxyquinoline	1.0	93
p-Hydroxymercuribenzoate	5.0	112
5,5'-Dithiobis(2-benzoate)	5.0	97
Iodoacetamide	1.0	111

APE (data not shown). The *K_m* for AsA was calculated to be 147 mM.

Effects of pH and temperature

The purified enzyme was active between pH 3 and 8. The maximum activity was obtained at pH 5.5 when ATP was used as the substrate, but it shifted to around pH 4.0 when PPI was used (Fig. 3). The optimal temperature was

Fig. 4. Effects of pH on Phosphatase activity.

The phosphatase activity was measured as described in Materials and Methods. The buffer used were (○, ●) sodium acetate buffer, (△, ▲) Tris maleate buffer, and (□, ■) Tris hydrochloride buffer. The substrate were *p*-NPP (A) and AsA2P (B).

50°C. When the enzyme was incubated for 30 min at various temperatures in 10 mM potassium phosphate buffer (pH 7.0), the original activity was retained at 50°C, but decreased to 67% and 0% at 60°C and 70°C, respectively. Purified APE was stable for several weeks when stored at 4°C in 10 mM PKB (pH 7.0) (data not shown).

Inhibitors

The effects of various agents on the enzyme activity are shown in Table IV. Among the divalent metal ions examined, Cu²⁺ inhibited the enzyme activity, but other divalent metal cations such as Ca²⁺, Mg²⁺, Fe²⁺, Mn²⁺ did not have any significant effects on the enzyme activity. The enzyme activity was not inhibited by sulfhydryl reagents, suggesting that APE contained no active-site thiol.

Phosphatase activity

The phosphatase activity with *p*-NPP or AsA2P as a substrate was detected at weakly acidic or neutral pH (Fig. 4). The *K_m* for *p*-NPP and AsA2P at pH 7.0 were 0.38 mM and 5.4 mM, respectively.

Discussion

We have found the phosphorylating activities of AsA in three bacteria including *P. azotocolligans* KY4661, as reported in our previous paper.¹⁰⁾ At that time, ATP was used

as a phosphoryl donor for the phosphorylation of AsA with either permeabilized cells or cell extracts, because it was thought to be catalyzed by a sort of kinase. The purified APE, however, used a variety of phosphoryl compounds as the donor besides ATP (Table III), indicating that it must be a phosphatase or phosphotransferase. In fact Tani *et al.* reported that the crystalline acid phosphatase of *Escherichia freundii* could catalyze the phosphorylation of pyridoxine.¹²⁾ Phosphorylation of sugars,¹³⁾ nucleosides,^{14,15)} and vitamins^{16,17)} were also reported to be catalyzed by phosphatase or phosphotransferase in several laboratories.

Therefore, we have studied the phosphatase activity of the purified APE. The *K_m* for *p*-NPP and AsA2P in the phosphatase activity were 0.38 mM and 5.4 mM, respectively, which were much lower than that for AsA, 147 mM in the phosphorylating reaction. The optimal pH for the AsA phosphorylation by APE was around 4.0 and 5.5 with PPI and ATP, respectively. Phosphatase of *E. freundii*,¹²⁾ and *P. mirabilis*¹⁷⁾ were reported to show the phosphorylating activities in a similar acidic range of pH.

Combined with these results, the conversion of AsA to AsA2P by APE might be done by the reversed reaction of its phosphatase activity. This is supported by the results that less than 50% of AsA could be phosphorylated even under the optimized conditions (data not shown).

Recently, Tani *et al.*^{18,19)} reported an enzyme activity in *Citrobacter freundii* that phosphorylated AsA mainly at the C-6 position. Our enzyme is different from theirs in that it could phosphorylate at the C-2 position specifically. To achieve more efficient production of AsA2P from AsA, the attempt

is being undertaken to clone the gene encoding this enzyme.

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