Manganese Ion-dependent Production of Phosphodiesterase by Alkalophilic *Bacillus* No. A-40-2 and Its Properties

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Phosphodiesterase production with bis-*p*-nitrophenyl phosphate as a substrate by alkalophilic *Bacillus* No. A-40-2 increased with increasing Mn^{2+} concentration, showing maximum productivity at 10 mM. The enzyme production was negligible in the medium without Mn^{2+} . The simultaneous addition of 10 mM Mn^{2+} and one of the several cations Mg^{2+} , Co^{2+} , Mo^{6+} , and Pb^{2+} at suitable concentrations stimulated the enzyme production 1.8-fold at most over that with only 10 mM Mn^{2+} . Inorganic phosphate hardly repressed the enzyme production. The enzyme was purified homogeneously. The purified enzyme had the optimum pH of 7.5 and was fairly stable from pH 7–11. The enzyme hydrolyzed 2',3'-cyclic-nucleotides and 3'-nucleotides, but did not hydrolyze 3',5'-cyclic-nucleotides or 5'-nucleotides, indicating it to be a 2',3'-cyclic-nucleotide 2'-phosphodiesterase (EC 3.1.4.16). The enzyme had activity without metals, but Mg^{2+} , Ca^{2+} , Ba^{2+} , and Mo^{6+} activated the enzyme reaction.

We reported on the manganese ion-dependent production of 5'-nucleotidase by alkalophilic *Bacillus*.¹⁾ Up to date, phosphodiesterase (2',3'-cyclic-nucleotide 2'-phosphodiesterase, EC 3.1.4.16) has been found in a number of strains of *Escherichia coli*,²⁾ *Enterobacteriaceae*,³⁾ *Vibrio alginolyticus*,⁴⁾ and *Bacillus subtilis*.^{5,6)} A variety of properties of phosphodiesterases of various organisms have been reported, but little has been reported on phosphodiesterase of alkalophilic microorganisms. Here we describe the extracellular production of phosphodiesterase by an alkalophilic *Bacillus* sp. depending on manganese ions in the growth medium, and the purification and some properties of the enzyme.

Materials and Methods

Culture conditions. A medium with the following composition was used as the standard medium (g/l): soluble starch, 15; $(\text{NH}_4)_2\text{SO}_4$, 5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.02; and NaHCO_3 , 10. The NaHCO_3 was sterilized separately. The microorganism was cultured in 50 ml of medium in a 300-ml two-necked flask after inoculation with 0.5 ml of overnight culture at 37°C on a rotary shaker (200 rpm).

Enzyme assay. Phosphodiesterase activity was measured by the amount of *p*-nitrophenol released from bis-*p*-nitrophenyl phosphate (BisPNPP) at 40°C in 15 min. The reaction mixture contained, in a total volume of 0.5 ml: 0.2 ml of 0.1 M HEPES-KOH buffer (pH 8.0), 0.01–0.05 ml of culture supernatant, 0.1 ml of 10 mM BisPNPP, and 0.15–0.19 ml of distilled water. The reaction was started by adding BisPNPP solution, stopped by the addition of 0.05 ml of 1 N NaOH, and diluted with 3.0 ml of distilled water. The absorbance at 410 nm was measured. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per min under these conditions.

Cyclic phosphodiesterase and nucleotidase were assayed at 40°C for 15 min by measuring the phosphate liberated from the respective substrates listed in Table IV, as stated above. Alkaline phosphatase with BisPNPP as a substrate, or alkaline phosphatase and purified 5'-nucleotidase of *Bacillus* No. A-59¹⁾ with 3',5'-cyclic-nucleotides were added to the reaction mixture. The reaction was stopped by the addition of 0.3 ml of 6 N H_2SO_4 . Phosphate was measured by the method of Fiske and Subarow, with some modifications, described previously.¹⁾ One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of inorganic phosphate per min.

Alkaline phosphatase was assayed by the previously described method.⁷⁾

Protein measurement. Protein was measured by the method of Lowry *et al.*,⁸⁾ with bovine serum albumin as standard protein.

SDS-Polyacrylamide gel electrophoresis. Electrophoresis was done at 30 mA for 2.5 hr at pH 8.3 using a SDS-PAGE plate 4/20 from Daiichi Pure Chemicals Co., Ltd. (Tokyo). The gel was stained with Coomassie brilliant blue.

Molecular weight measurement. The molecular weight of the phosphodiesterase was measured by the method of Andrew⁹⁾ on a column (1.5 × 100 cm) of Cellulofine GCL-1000-sf which had been equilibrated with 0.05 M Tris-HCl buffer, pH 7.2.

Chemicals. Cyclic nucleotides and mononucleotides were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and yeast extract from Difco Laboratories (Detroit, Mich., U.S.A.). All other chemicals were of reagent grade.

Results

Mn²⁺-dependent production of phosphodiesterase

Of the alkalophilic strains isolated in our laboratory, No. A-40-2,¹⁰⁾ an alkaline amylase producer, was chosen due to its phosphodiesterase productivity. Phosphodiesterase was found mainly in the culture supernatant and slightly in the cell-free extract. The enzyme activity levelled off between 20–35 hr of cultivation in the standard medium. The enzyme production increased with increasing Mn^{2+} concentration, with maximum productivity at 10 mM. As shown in Fig. 1, phosphodiesterase production was negligible in the medium without Mn^{2+} ($MnCl_2$ -omitted standard me-

dium). The other cations Ca^{2+} , Ba^{2+} , Mg^{2+} , Co^{2+} , Fe^{2+} , Mo^{6+} , Zn^{2+} , Ni^{2+} , or Pb^{2+} , added singly at 0.1, 1.0, or 10 mM, had no stimulatory effect on phosphodiesterase production. Alkaline phosphatase production that was observed in the standard medium was not increased with increasing Mn^{2+} .

Effects of simultaneous addition of Mn^{2+} and other cations

The enzyme activity was assayed after the culture fluid was dialyzed against water for 5 hr. The simultaneous addition of 10 mM Mn^{2+} and one of the several cations Mg^{2+} , Co^{2+} , Mo^{6+} and Pb^{2+} stimulated the phosphodiesterase production 1.8-fold at most (Table I). The other cations, Ca^{2+} , Ba^{2+} , Fe^{2+} , and Zn^{2+} , added simultaneously with 10 mM Mn^{2+} , repressed phosphodiesterase production slightly or appreciably.

Effects of inorganic phosphate on the enzyme production

As shown in Table II, 1 mM K_2HPO_4 increased the enzyme production to a certain extent and 10 mM K_2HPO_4 repressed the enzyme production only by 15%.

Purification and molecular weight of phosphodiesterase

All purification procedures were done below

Fig. 1. Stimulatory Effect of Mn^{2+} on the Production of the Phosphodiesterase.

The enzyme activity was assayed after 24 hr of cultivation. □, growth; ■, enzyme activity.

5°C. The enzyme solution was concentrated with a Diaflow membrane PM 10 throughout the purification. Table III summarizes the purification. The culture supernatant after

Table I. EFFECTS OF SIMULTANEOUS ADDITION OF Mn^{2+} AND VARIOUS METALS ON PHOSPHODIESTERASE PRODUCTION

Metal added	Concentration (mM)	Growth (A_{600})	Enzyme activity (units/ml)
None	—	24.5	0.478
$MgCl_2$	5	27.6	0.559
	10	27.5	0.541
	20	26.2	0.627
	30	25.7	0.566
$Co(NO_3)_2$	0.05	24.8	0.501
	0.2	23.9	0.844
	1	18.6	0.459
Na_2MoO_4	5	8.7	0.022
	5	20.4	0.714
	10	19.8	0.841
	20	19.4	0.857
$PbCl_2$	0.1	20.3	0.650
	1	6.5	0.034

Each medium contained 10 mM Mn^{2+} . The enzyme activity was assayed after 24 hr of cultivation.

Table II. EFFECTS OF INORGANIC PHOSPHATE ON THE PHOSPHODIESTERASE PRODUCTION IN THE PRESENCE OF 10 mM Mn^{2+}

K_2HPO_4 concentration (mM)	Growth (A_{600})	Enzyme activity (units/ml)	Relative activity (%)
0	21.9	0.514	100
0.1	20.5	0.523	102
1	18.0	0.613	119
10	15.7	0.436	85

The enzyme activity was assayed after 24 hr of cultivation.

24 hr of cultivation with 10 mM Mn^{2+} was concentrated to about one fifth of the initial volume with polyethyleneglycol 6000. The concentrated enzyme solution was dialyzed for 2 days against 0.05 M Tris-HCl buffer, pH 8.0, with a change of the buffer solution. The dialyzed solution was put on a DEAE-Toyopearl 650 M column (1.5 × 40 cm) equilibrated with the buffer described above. After the column had been washed with the same buffer, the enzyme was eluted with a linear gradient of NaCl (0–0.5 M) in the equilibration buffer (total volume 300 ml). The active fraction (35 ml) was concentrated and dialyzed against the equilibration buffer. The second DEAE-Toyopearl chromatography was done by the same procedure. For the gel filtration experiment, the concentrated enzyme solution was put on a column (1.5 × 40 cm) of Sephacryl S-300 equilibrated with 0.05 M Tris-HCl buffer (pH 8.0) containing 0.05 mM $Co(NO_3)_2$, which stabilized the enzyme at low temperatures for a long time. The homogeneity of the purified enzyme was confirmed by the SDS-

Fig. 2. Electrophoretic Profile of the Enzyme in an SDS-Polyacrylamide gel.

The gel was stained with Coomassie brilliant blue.

Table III. SUMMARY OF THE PURIFICATION

	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Recovery (%)
Culture fluid	11.09	170.1	0.0652	100
Polyethyleneglycol and dialysis	7.98	36.5	0.219	72
First DEAE-Toyopearl	7.43	1.81	4.10	67
Second DEAE-Toyopearl	6.76	1.12	6.04	61
Sephacryl S-300	5.65	0.437	12.93	51

Fig. 3. pH Stability of the Phosphodiesterase.

The enzyme was treated in each buffer at 40°C for 15 min. After the pH had been adjusted to 8.0 with an HCl or KOH solution, the residual activity was measured by the standard assay method. The buffer systems were as follows: 0.1 M HEPES-KOH (pH 5.0–8.5, ▲), 0.1 M KHCO₃-K₂CO₃ (pH 8.5–11.0, ■), and 0.1 M K₂CO₃-KOH (pH 12.0 and 13.0, □).

polyacrylamide gel electrophoresis shown in Fig. 2. Its molecular weight estimated by gel filtration on Cellulofine GCL-1000-sf column (1.5 × 100 cm) was 122,000 and that by SDS-PAGE was 120,000.

Effects of pH and temperature on activity and stability

The optimum pH for the enzyme activity was 7.5. Figure 3 shows the pH stability. The enzyme was fairly stable over the pH range of 7–11. The enzyme had an optimum temperature of 40–45°C in the pH 7.5 HEPES-KOH buffer for 15 min of incubation, and had 100% activity at 35°C after treatment for 15 min at pH 7.5.

Substrate specificity

We investigated the phosphodiesterase with BisPNPP as a substrate, but there are several types of phosphodiesterases by their specificities. Table IV shows the relative activity for different kinds of nucleotides. The enzyme of this strain strongly hydrolyzed 2',3'-cyclic-nucleotides and 3'-nucleotides, but did not hydrolyze 3',5'-cyclic-nucleotides and 5'-nucleotides, indicating that the enzyme is a 2',3'-cyclic-nucleotide 2'-phosphodiesterase (EC

Table IV. ACTIVITY OF PHOSPHODIESTERASE TOWARD VARIOUS SUBSTRATES

Substrate	Relative activity (%)
BisPNPP	100
2',3'-cyclic AMP	278
2',3'-cyclic CMP	245
2',3'-cyclic GMP	253
2',3'-cyclic UMP	433
3',5'-cyclic AMP	0
3',5'-cyclic CMP	0
3',5'-cyclic GMP	0
3',5'-cyclic UMP	0
3'-AMP	217
3'-CMP	218
3'-GMP	404
3'-UMP	451
2'-AMP	0
2'-GMP	0
5'-AMP	0
5'-CMP	0
5'-GMP	0
5'-UMP	0
5'-dAMP	0
5'-dGMP	0
ADP	2
ATP	0
p-Nitrophenyl phosphate	0

3.1.4.16). The Michaelis constant and $V_{\max}$ for BisPNPP were 1.2 mM and 35 $\mu\text{mol}/\text{min}/\text{mg}$ protein, respectively.

Effects of various reagents

A mixture (0.4 ml) consisting of 0.02 ml of the enzyme solution dialyzed against distilled water, 0.2 ml of 0.1 M HEPES-KOH buffer (pH 8.0), 0.01–0.1 ml of metal ion or EDTA solution, and 0.08–0.17 ml of distilled water was incubated at 40°C for 15 min. Then 0.1 ml of the substrate was added. Table V summarizes the effects of reagents on enzyme activity. The enzyme had activity without metals, but Mg^{2+} , Ca^{2+} , Ba^{2+} , and Mo^{6+} activated the enzyme reaction. EDTA slightly affected the enzyme activity.

Discussion

The extracellular production of a phosphodiesterase by alkalophilic *Bacillus* was en-

Table V. EFFECTS OF VARIOUS COMPOUNDS ON THE HYDROLYSIS OF PHOSPHODIESTERASE

Compound	Concentration (mM)	Activity (%)
None	—	100
MgCl ₂	1	103
	5	118
	10	125
	20	120
CaCl ₂	1	124
	5	127
	10	132
	20	149
BaCl ₂	0.1	113
	1	141
	5	136
Na ₂ MoO ₄	10	119
	30	127
	50	126
MnCl ₂	0.01	102
	0.1	78
Co(NO ₃) ₂	0.01	99
	0.1	82
FeSO ₄	0.1	76
ZnSO ₄	0.1	7
NiSO ₄	0.1	69
PbCl ₂	0.1	82
CuSO ₄	0.01	8
CdSO ₄	0.1	41
SnCl ₂	0.1	88
EDTA	0.1	82
	1.0	79

hanced by the addition of a high concentration, 10 mM, of Mn²⁺ to the culture medium. Several cations stimulated the enzyme production when added simultaneously with 10 mM Mn²⁺. Ammonium sulfate was used as a nitrogen source because of its efficiency (data not shown). The role of a high concentration of Mn²⁺, 10 mM, in the enzyme production was not understandable exactly, but it may be involved in the triggering of the enzyme synthesis as in the case of 5'-nucleotidase.¹⁾ There has been no report on stimulatory effects of Pb²⁺ on enzyme production, and it is of interest that high concentrations of Mo⁶⁺,

5–20 mM, stimulated enzyme production (Table II). In many cases, inorganic phosphate was added to the culture media for the production of phosphodiesterase, indicating that phosphodiesterase is constitutive. In this strain, the Mn²⁺-stimulated enzyme production was scarcely affected by inorganic phosphate.

The molecular weight of 120,000 was larger than those reported for other phosphodiesterases.¹¹⁾ The optimum pH of 7.5 was similar to those of enzymes reported for neutrophilic strains.¹¹⁾ The thermostability range of pH 7–11 shows a characteristic feature of the enzyme from an alkalophilic strain, although there has been no paper on the pH stability of phosphodiesterases. The optimum temperature and thermostability data shows the similarity to other phosphodiesterases. Several types of phosphodiesterases were known, and phosphodiesterase of this strain belongs to 2',3'-cyclic-nucleotide 2'-phosphodiesterase (EC 3.1.4.16) due to its substrate specificity. Cobalt ion stabilized the enzyme at 5°C for a long time (> 1 week), but it was not effective at 40°C.

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