Purification and Properties of Alkaline Phosphodiesterase from the Fruit Body of *Flammulina velutipes*

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An alkaline phosphodiesterase has been purified 2200-fold from the fruit body of *Flammulina velutipes*. The molecular weight is about 53,000. The optimum pH for hydrolysis of bis-p-nitrophenyl phosphate is 8.0. This phosphodiesterase is stable for 30 min below 40°C and in a pH range from 5.5 to 6.5. The $K_m$ values for bis-p-nitrophenyl phosphate and $p$-nitrophenyl deoxothymidine 5'-phosphate are 0.25 mM and 0.09 mM, respectively. The enzyme does not hydrolyze RNA and DNA, whereas adenosyl uridine 5'-phosphate is hydrolyzed at a considerable rate. The phosphodiesterase also hydrolyzes NAD, UDP-glucose, and ADP-ribose but does not act on ATP and cyclic AMP.

It is generally recognized that organisms have a set of various kinds of nucleolytic enzymes in their cells. Each enzyme has a special mode of action with its own substrate specificity. The nucleolytic system in *E. coli* has been intensively studied by many workers and several kinds of enzymes have been found to function in different manners. In the interest of comparative biochemistry, a systematic study of nucleolytic enzymes in basidiomycetes has been carried out in our laboratory.

*Flammulina velutipes* is a basidiomycete which can produce a fruit body in its life cycle. In the previous paper,\(^1\) we reported the presence of ATP-sensitive and ATP-insensitive ribonucleases in the fruit body of *F. velutipes*. A phosphodiesterase activity judged with bis-\(p\)-nitrophenyl phosphate as substrate was also detected in the same preparation. This paper describes a purification procedure of the phosphodiesterase (phosphoric diester hydrolase EC 3.1.4) from the fruit body of *F. velutipes* and some molecular and catalytic properties of the enzyme are also presented.

### MATERIALS AND METHODS

**Materials.** Fresh fruit bodies of *Flammulina velutipes* were purchased from a mushroom farmer. Caps of fruit bodies were used for enzyme preparation.

**Substrates.** Nitrophenyl-pT, Tp-nitrophenyl, ApU, ApA, UDP-glucose, ADP-ribose, ATP, 2',3'-cyclic AMP, 3',5'-cyclic AMP, 3',5'-cyclic GMP, and 5'-AMP were purchased from Boehringer Mannheim GmbH, yeast RNA and ADP from the Sigma Chemical Co., and bis-\(p\)-nitrophenyl phosphate and disodium \(p\)-nitrophenyl phosphate from Nakarai Chemicals Ltd. RNA was further purified by means of phenol treatment and alcohol precipitation followed by extensive dialysis against distilled water.

**Other reagents.** DEAE-cellulose was purchased from Whatman Biochemicals Ltd., CM-Sephadex C-50 from Pharmacia Fine Chemicals, cellulose thin layer plate (Avicel SF) from the Funakoshi Chemicals Co., 2'(3')-AMP and adenosine from Kohjin Co., Ltd., uridine from the Seikagaku Kogyo Co., Ltd., alkaline phosphatase (EC 3.1.3.1, calf intestine), cytochrome c, chymotrypsinogen A, hen egg albumin, and bovine serum albumin from Boehringer Mannheim GmbH.

**Enzyme assay.** Enzyme activities were routinely estimated by assay 1 at each of the purification steps using bis-\(p\)-nitrophenyl phosphate as substrate.

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Abbreviations: PDase, phosphodiesterase; PMase, phosphomonoesterase; RNase, ribonuclease; DNase, deoxyribonuclease; Nitrophenyl-pT, \(p\)-nitrophenyl deoxothymidine 5'-phosphate; Tp-nitrophenyl, \(p\)-nitrophenyl deoxothymidine 3'-phosphate; ApU, adenosyl uridine 5'-phosphate; ApA, adenosyl adenosine 5'-phosphate.
Assay 1. Enzyme activity toward bis-p-nitrophenyl phosphate was determined by measurement of the amount of p-nitrophenol released. The standard reaction mixture contained 0.5 mM bis-p-nitrophenyl phosphate, 50 mM Tris-HCl buffer, pH 8.0, and enzyme in a total volume of 1.0 ml, and was incubated for 30 min at 37°C. The reaction was stopped by addition of 2 ml of 0.1 N NaOH solution and the absorbance at 400 nm was measured against a suitable blank. PMase activity was estimated essentially in the same manner using disodium p-nitrophenyl phosphate as substrate. Enzyme activity toward nitrophenyl-pT was determined by the above method except that the substrate concentration was varied as indicated in the figure legend. One unit of enzyme was defined as the amount which liberated 1 μmol of p-nitrophenol per 1 min.

Assay 2. Enzyme activities toward nucleoside mono-, di-, and triphosphate were determined by measurement of the amount of inorganic phosphate released. The reaction mixture contained 2 mM substrate, 50 mM Tris-HCl buffer, pH 8.0, and enzyme in a total volume of 0.5 ml and was incubated for 30 min at 37°C. The reaction was stopped by addition of 0.1 ml of ice-cold 25% perchloric acid and the inorganic phosphate released was estimated by Deniges’s method.

Assay 3. Enzyme activities toward ApU, ApA, NAD, UDP-glucose, ADP-ribose, 2',3'-cyclic AMP, 3',5'-cyclic AMP, and 3',5'-cyclic GMP were determined by measurement of the amount of inorganic phosphate released by hydrolysis of the nucleoside monophosphate by alkaline phosphatase. The reaction mixture contained 2 or 4 mM substrate, 50 mM Tris-HCl buffer, pH 8.0, 5 mM magnesium acetate, 0.5 unit alkaline phosphatase, and enzyme in a total volume of 0.5 ml and was incubated for 30 min at 37°C. The reaction was stopped by addition of 0.1 ml of ice-cold 25% perchloric acid and the mixture was allowed to stand for 20 min in the cold. Then an aliquot was taken for determination of inorganic phosphate.

Assay 4. RNase and DNase activities were estimated by measuring optical density at 260 nm of the acid-soluble degradation product using yeast RNA and heat-denatured calf thymus DNA as substrates, respectively.

Polyacrylamide slab gel electrophoresis. Polyacrylamide slab gel electrophoresis was carried out in a 10.5% slab gel (2 mm thick) with Tris-HCl buffer, pH 9.4. One half of the gel was stained with Coomassie Brilliant Blue R-250 for protein. The other half was used to estimate the enzyme activity.


Determination of protein content. Protein was determined according to the method of Lowry et al. with crystalline bovine serum albumin as a standard material. The optical density at 280 nm was also measured at each of the purification steps.

RESULTS

Purification of Phosphodiesterase

All operations were carried out between 0~4°C unless otherwise stated.

Step 1. Crude extract. Caps of F. velutipes (wet weight 1700 g) were homogenized with 3.5 liters of 0.02 M phosphate buffer, pH 6.0, in a dispersing mixer (MRK Co., Ltd.). The homogenate was centrifuged at 8500 × g for 30 min and the supernatant fluid (4.0 liters) was used as the crude enzyme extract.

Step 2. Ammonium sulfate fractionation. The supernatant fluid was brought to 45% saturation by adding 25.8 g of solid ammonium sulfate per 100 ml of solution with stirring. After standing overnight, the precipitate was removed by centrifugation at 8500 x g for 45 min. This fractionation resulted in the removal of glutinous substances. The supernatant fluid was brought to 100% saturation by adding 38.3 g of solid ammonium sulfate per 100 ml of the solution. After standing overnight, the precipitate was collected by centrifugation at 8500 × g for 30 min and dissolved in 500 ml of 0.02 M phosphate buffer, pH 6.0. The solution was dialyzed against the same buffer with two changes of 5 liters each overnight.

Step 3. Chromatography on DEAE-cellulose (pH 6.0). Dialyzed enzyme solution (640 ml) was applied to a DEAE-cellulose column (DE 23, 4.4 × 26 cm) previously equilibrated with 0.02 M phosphate buffer, pH 6.0. The column was washed with 1500 ml of the above buffer. Elution was carried out with a linear gradient formed with 750 ml each of 0.1 and 0.5 M NaCl in 0.02 M phosphate buffer, pH 6.0. Fractions of 10 ml were collected at a flow rate of 80 ml/hr. ATP-sensitive RNase came directly through the column. PMase was eluted at 0.26 M NaCl. PMase was eluted at 0.18 M NaCl. RNase was eluted in two peaks of activity; one was at 0.15 M and the other at
0.21 M NaCl. The latter RNase fraction was accompanied by DNase activity. The active fractions (430 ml) were combined and concentrated to about 40 ml by ultrafiltration. The enzyme solution was then dialyzed against 0.02 M phosphate buffer, pH 6.0, with two changes of 3 liters each overnight.

**Step 4. Chromatography on DEAE-cellulose (pH 6.0).** The enzyme preparation from Step 3 was applied to a DEAE-cellulose column (DE 32, 2.2 × 16 cm) previously equilibrated with 0.02 M phosphate buffer, pH 6.0, containing 0.07 M NaCl. Elution was carried out with a linear gradient formed with 250 ml each of 0.07 and 0.35 M NaCl in 0.02 M phosphate buffer, pH 6.0. Fractions of 5 ml were collected at a flow rate of 35 ml/hr. Active fractions (155 ml) were combined and concentrated to about 20 ml, then dialyzed against 0.02 M phosphate buffer, pH 7.5, with two changes of 3 liters each.

**Step 5. Chromatography on DEAE-cellulose (pH 7.5).** The enzyme preparation from Step 4 was applied to a DEAE-cellulose column (DE 32, 1.6 × 13 cm) previously equilibrated with 0.02 M phosphate buffer, pH 7.5. Elution was carried out with a linear gradient formed with 200 ml each of 0 and 0.3 M NaCl in 0.02 M phosphate buffer, pH 7.5. Fractions of 4 ml were collected at a flow rate of 30 ml/hr. As shown in Fig. 1, PDase was eluted at about 0.19 M NaCl. The active fractions (104 ml) were combined and concentrated to about 10 ml, then dialyzed against 0.02 M acetate buffer, pH 5.5, with two changes of 2 liters each.

**Step 6. Chromatography on CM-Sephadex C-50.** The enzyme preparation from Step 5 was applied to a CM-Sephadex C-50 column (1.6 × 25 cm) previously equilibrated with 0.02 M acetate buffer, pH 5.5, containing 0.05 M NaCl. The latter RNase fraction was accompanied by DNase activity. The active fractions (430 ml) were combined and concentrated to about 40 ml by ultrafiltration. The enzyme solution was then dialyzed against 0.02 M phosphate buffer, pH 6.0, with two changes of 3 liters each overnight.

### Table 1. Summary of Purification of Alkaline Phosphodiesterase

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (unit/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>15450</td>
<td>54.7</td>
<td>0.0035 (1.0)</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate (45~100%)</td>
<td>1033</td>
<td>40.2</td>
<td>0.0389 (11.1)</td>
<td>73.5</td>
</tr>
<tr>
<td>1st DEAE-cellulose (pH 6.0)</td>
<td>288</td>
<td>31.3</td>
<td>0.111 (31.7)</td>
<td>57.2</td>
</tr>
<tr>
<td>2nd DEAE-cellulose (pH 6.0)</td>
<td>73.9</td>
<td>25.4</td>
<td>0.344 (98.3)</td>
<td>46.4</td>
</tr>
<tr>
<td>3rd DEAE-cellulose (pH 7.5)</td>
<td>43.0</td>
<td>19.5</td>
<td>0.453 (129.4)</td>
<td>35.6</td>
</tr>
<tr>
<td>CM-Sephadex C-50</td>
<td>0.98</td>
<td>7.5</td>
<td>7.653 (2186.6)</td>
<td>13.7</td>
</tr>
</tbody>
</table>

Details are described in the text. ○, PDase activity; ●, absorbance at 280 nm; ———, NaCl concentration.
NaCl. Stepwise elution was carried out with 0.05 M and 0.3 M NaCl in the above buffer. Fractions of 3 ml were collected at a flow rate of 10 ml/hr. About 98% of the protein applied to the column was eluted at 0.05 M NaCl and slight enzyme activity was detected in this fraction. The major part of the PDase activity emerged at 0.3 M NaCl (Fig. 2). The fractions of 18~21 (12 ml) were pooled and dialyzed against 0.02 M phosphate buffer, pH 6.0, with two changes of 2 liters each. This enzyme preparation was used for examination of several properties.

**Purity of the enzyme**

The purity of the enzyme from Step 6 was examined by polyacrylamide slab gel electrophoresis. As shown in Fig. 3, two protein bands were detected. The activity for ApA and nitrophenyl-pT coincided with the band which moved faster. The activity for ADP-ribose also existed in this band. Another band showed no activity for the substrates described above.

**Properties of *F. velutipes* PDase**

**Molecular weight.** The molecular weight of PDase was estimated by gel filtration on Sephadex G-75 with appropriate molecular weight standards by the method of Andrews.

As shown in Fig. 4, the molecular weight estimated was about 53,000.

**Catalytic properties**

**Effect of pH.** The effect of pH on the enzyme activity is shown in Fig. 5. The enzyme shows a sharp pH optimum of 8.0 for bis-p-nitrophenyl phosphate. After preincubation of the enzyme at various pH for 30 min at 45°C, the remain-

![Fig. 4. Estimation of the Molecular Weight of PDase by Gel Filtration.](image)

The enzyme was applied to a Sephadex column (2.0 x 75 cm) equilibrated with 0.02 M phosphate buffer, pH 6.0. Elution was carried out with the same buffer. Fractions of 3 ml were collected at a flow rate of 10 ml/hr. PDase was detected by the standard assay method. The molecular weights of the standard proteins used are as follows: cytochrome c, 12,500; chymotrypsinogen A, 25,000; hen egg albumin (HEA), 45,000; bovine serum albumin (BSA), 67,000.

![Fig. 5. Effect of pH on the Activity of PDase.](image)

Activity was measured with bis-p-nitrophenyl phosphate as substrate by assay 1 except that the pH of the buffer was varied as indicated. The buffer solutions used were: 0.2 M sodium acetate buffer, 0.2 M phosphate buffer, 0.2 M Tris-HCl buffer.
Heat stability. The effect of temperature on the enzyme stability at pH 6.0 is shown in Fig. 6. The enzyme was stable up to 40°C and the activity was completely lost after treatment at 50°C for 15 min.

Effect of metal ions and other compounds. The metal ion requirement of the enzyme activity with bis-\(p\)-nitrophenyl phosphate as substrate was examined. EDTA and o-phenanthroline caused 91% and 99% inhibition, respectively. Among various metal ions tested at 1 mM, none stimulated the enzyme activity except that \(\text{Zn}^{2+}\) at a low concentration (50 \(\mu\)M) stimulated the enzyme activity by 23%. Dialysis of the enzyme against 0.02 M Tris-HCl buffer containing 1 mM EDTA caused a nearly complete inactivation of the enzyme. The activity of the EDTA-treated enzyme was restored to the level of 30% of the initial activity by incubation with 50 \(\mu\)M \(\text{Zn}^{2+}\), indicating that the enzyme was very unstable in the absence of \(\text{Zn}^{2+}\). PCMB did not cause any inactivation of the enzyme.

Kinetics of enzyme reaction. The effects of bis-\(p\)-nitrophenyl phosphate and nitrophenyl-pT concentration on the reaction velocities were examined. The reaction velocities were measured by assay 1. The plots showed a straight line in either case (Fig. 7). \(K_m\) values were calculated to be 0.25 mM for bis-\(p\)-nitrophenyl phosphate and 0.09 mM for nitrophenyl-pT.

Substrate specificity

The enzyme activity was examined with a number of substrates. The results are summarized in Table II.

Synthetic substrates. Bis-\(p\)-nitrophenyl phosphate, nitrophenyl-pT, and Tp-nitrophenyl were hydrolyzed. Nitrophenyl-pT was the most effective substrate and it was hydrolyzed 16 times as fast as Tp-nitrophenyl.
Table II. Substrate Specificity of Alkaline Phosphodiesterase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity*</th>
<th>Assay method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrophenyl-pT</td>
<td>272</td>
<td>1</td>
</tr>
<tr>
<td>Tp-Nitrophenyl</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>Bis-p-nitrophenyl phosphate</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>p-Nitrophenyl phosphate</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2',3'-Cyclic AMP</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>3',5'-Cyclic AMP</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>3',5'-Cyclic GMP</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>ApU</td>
<td>47</td>
<td>3</td>
</tr>
<tr>
<td>ATP</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>ADP</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>5'-AMP</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>NAD</td>
<td>52</td>
<td>3</td>
</tr>
<tr>
<td>UDP-Glucose</td>
<td>23</td>
<td>3</td>
</tr>
<tr>
<td>ADP-Ribose</td>
<td>86</td>
<td>3</td>
</tr>
</tbody>
</table>

* The enzyme activity for bis-p-nitrophenyl phosphate was taken as 100%.

When the reaction mixture for Tp-nitrophenyl was incubated in the absence or presence of alkaline phosphatase, the activity measured was the same in either case. This result indicates that p-nitrophenyl phosphate was not produced from Tp-nitrophenyl. The production of p-nitrophenyl phosphate from nitrophenyl-pT was not observed either.

Nucleotides. ATP, ADP, and all the nucleoside monophosphate tested were not hydrolyzed. No enzyme activity was found toward cyclic nucleotides such as 2',3'-cyclic AMP, 3',5'-cyclic AMP, or 3',5'-cyclic GMP under the assay conditions used.

Dimucleotides. ApU was hydrolyzed at a considerable rate. ApU was split mainly to 5'-UMP and adenosine when examined on cellulose thin layer chromatography with solvent system A. The molar ratio of 5'-UMP and adenosine was 1.0.

Pyrophosphates. The enzyme also catalyzed the cleavage of the pyrophosphate bonds of UDP-glucose, NAD, and ADP-ribose. 5'-UMP and 5'-AMP were detected on the silica gel thin layer chromatogram with solvent B of the digestion products from UDP-glucose and NAD, respectively. It is therefore considered that UDP-glucose was split to 5'-UMP and glucosyl monophosphate and that NAD was split to 5'-AMP and nicotinamide mononucleotide. Similarly, ADP-ribose was shown to be split to 5'-AMP and ribosyl monophosphate.

DISCUSSION

The enzyme was purified about 2200-fold over the crude extract. Fractionation of the crude extract by 45% ammonium saturation was an essential and very effective procedure to remove the glutinous substances of mushrooms. DEAE-cellulose column chromatography at steps 3 and 4 resulted in the effective removal of other nucleolytic enzymes and PMase which existed in the crude extract. When examined on a polyacrylamide slab gel, the enzyme preparation at step 6 was shown to be contaminated by at least one protein species. However, the results in Fig. 3 indicate that the activity of hydrolyzing the phosphodiester and pyrophosphate bonds is attributed to the same protein. The apparent molecular weight of the enzyme was estimated to be 53,000 by gel filtration. This value is very small in comparison with that (130,000) of snake venom PDase,4) a representative PDase. The molecular weights of the alkaline PDases of plants were reported to be 100,000 and 110,000 for the carrot5) and sugar beet leaves,6) respectively.

PDases from edible mushrooms have been described by other investigators. The enzyme from Lentinus edodes7) has a pH optimum of about 7.5, while Tricholoma matsutake8) contains an acid PDase which shows the maximal activity at pH 6.0 to 6.5. The alkaline PDase of L. edodes remained partially active after incubation at 70°C for 15 min in contrast to the heat-lability of F. velutipes enzyme (Fig. 6).

The enzyme does not hydrolyze polynucleotides such as yeast RNA and heat-denatured calf thymus DNA, indicating that the enzyme is not a type of exonuclease. Among the synthetic substrates tested, nitrophenyl-pT was the most effective sub-
strate which was hydrolyzed 16 times as fast as Tp-nitrophenyl. In contrast, tobacco acid PDase shows the same reaction velocities to both nitrophenyl-pT and Tp-nitrophenyl.\(^9\) The above result suggests that \textit{F. velutipes} PDase produces 5'-mononucleotides preferentially. This is supported by the mode of action on ApU, which is split to produce adenosine and 5'-UMP preferentially. The formation of small amounts of 3'-AMP and uridine from ApU, though a quantitative analysis is not yet achieved, may be ascribed to a little activity of PDase toward Tp-nitrophenyl.

As shown in Fig. 7, \(K_m\) values for bis-p-nitrophenyl phosphate and nitrophenyl-pT are 0.25 mM and 0.09 mM, respectively. \(K_m\) values of PDases from snake venom\(^10\) and carrot\(^11\) for nitrophenyl-pT are 0.5 mM and 0.02 mM, respectively. For nitrophenyl-pU, the alkaline PDase of sugar beet leaves\(^6\) have \(K_m\) value of 0.19 mM. The acid PDase of cultured tobacco cells has two \(K_m\) values of 0.17 mM and 1.3 mM.\(^9\)

The enzyme split pyrophosphate bonds; UDP-glucose, NAD, and ADP-ribose were hydrolyzed at a considerable rate. However, the enzyme showed no activity on ATP and ADP. Its inability to attack ATP and ADP is a remarkable property which distinguishes \textit{F. velutipes} PDase from the other known PDases\(^5,6,9,12\) except for rat liver PDase.\(^13\)

It was reported that cyclic AMP plays an important role in the formation of the fruit body of \textit{Coprinus macrorhizus} and the amount of cyclic AMP is controlled by cyclic nucleotide PDase.\(^14\) The PDase of \textit{F. velutipes} showed no activity on 3',5'-cyclic AMP and 3',5'-cyclic GMP, indicating that it does not participate in the metabolism of cyclic nucleotides. It is probable that another enzyme which is responsible for the digestion of cyclic nucleotides occurs in the cells of \textit{F. velutipes}.

From the results obtained here, the enzyme of the present study resembles the PDase of rat liver\(^13\) in several respects as follows. a) Both alkaline PDases are heat-labile and sensitive to EDTA. b) Both hydrolyze oligonucleotides to produce 5'-nucleotides at a considerable rate in spite of their very low or absent activity toward DNA and RNA. c) Both split the pyrophosphate bond such as NAD but cannot act on ATP and ADP. A detailed study on the oligonucleotidase activity of the PDase from \textit{F. velutipes} will be published in the near future.

REFERENCES