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Hydrolysis of Some Chromogenic Substrates and Adenosine Triphoshate Catalyzed by Phosphodiesterase-phosphomonoesterase from *Fusarium moniliforme*

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For comparison with known phosphodiesterases, the mode of action of phosphodiesterase-phosphomonoesterase from *Fusarium moniliforme* toward some key substrates was examined. The enzyme catalyzed the hydrolysis of either thymidine 3′-(4-nitrophenylphosphate) or thymidine 5′-(4-nitrophenylphosphate). The former substrate was hydrolyzed processively into thymidine, inorganic phosphate and 4-nitrophenol, whereas the latter was split into 4-nitrophenol and thymidine 5′-phosphate. The kinetic constants determined on the basis of 4-nitrophenol liberation were: $K_m=4.6$ mmol dm^{-3} and $k_{cat}=115$ s^{-1} for thymidine 3′-(4-nitrophenylphosphate); $K_m=0.46$ mmol dm^{-3} and $k_{cat}=19.7$ s^{-1} for thymidine 5′-(4-nitrophenylphosphate) at pH 5.3 and 37 °C. 4-Nitrophenyl phenylphosphonate was a good substrate for the enzyme showing kinetic constants $K_m=1.4$ mmol dm^{-3} and $k_{cat}=360$ s^{-1} at pH 5.3 and 37 °C. However, its chiral thio analogue, *O*-(4-nitrophenyl) phenylphosphonothioate, was hydrolyzed by the enzyme only very slowly in a stereo-nonspecific manner. The three phosphoryl groups in adenosine triphosphate was removed by the enzyme sequentially from the terminal position. These findings have made the uniqueness of *Fusarium* phosphodiesterase-phosphomonoesterase clearer.

Snake venom phosphodiesterase and spleen phosphodiesterase are representatives of many phosphodiesterases so far known. Although the classification of these two enzymes is based on the mode of hydrolysis of polynucleotides, they can easily be distinguished by the use of artificial chromogenic substrates; thymidine 3′-(4-nitrophenylphosphate) (TpONph) and thymidine 5′-(4-nitrophenylphosphate) (NphOpT). Snake venom phosphodiesterase is active only with the latter, whereas spleen phosphodiesterase is active only with the former.[1]

Kelly *et al.*[2] introduced a new chromogenic substrate, 4-nitrophenyl phenylphosphonate (PhPONph) for distinguishing these two phosphodiesterases. They showed that PhPONph is hydrolyzed only by phosphodiesterases of snake venom type (5′-nucleotide phosphodiesterases according to their terminology). Dudman and Benkovic[3] showed that the thio analogue of PhPONph, *O*-(4-nitrophenyl) phenylphosphonothioate (PhP(S)-ONph), is hydrolyzed by phosphodiesterases of the same type. PhP(S)ONph has a chiral phosphorus center. Snake venom phosphodiesterase hydrolyzes specifically the S_p diastereomer.[4]

Phosphodiesterase-phosphomonoesterase (PDMase) of the phytopathogenic fungus, *Fusarium moniliforme*, is a unique phosphohydrolase active with either phosphoric diesters or phosphoric monoesters. Yoshida *et al.*[5] pointed out that PDMase resembles snake venom phosphodiesterase, because a diribonucleoside monophosphate is first split by PDMase into a nucleoside and a 5′-nucleotide. Thus it is of interest to compare PDMase with snake venom phosphodiesterase more precisely using the above chromogenic substrates. This paper describes the action of PDMase on these substrates along with adenosine triphosphate (ATP).

Experimental

Materials. TpONph and NphOpT were purchased from Sigma and Calbiochem, respectively. Snake venom phosphodiesterase of *Crotalus adamanteus* was the product of Sigma (Type II), the activity of which was assayed at 37 °C with 1 mmol dm^{-3} bis(4-nitrophenyl) phosphate as a substrate in 50 mmol dm^{-3} Tris–HCl buffer (pH 8. 8) containing 1 mmol dm^{-3} $MgCl_2$. One unit (U) of activity was defined as that amount which liberates 1 μmol of 4-nitr ophenol per min under the above conditions. PDMase was purified from dried powder of the culture filtrate of *F. moniliforme* (commercial name, Toyocelase A, Toyo Brewery Co.) as described previously.[6] An isozyme mixture was used, because no difference in enzymic properties had so far been found among the isozymes.[7] PDMase was determined on the basis of activity toward 4-nitrophenyl phosphate. The assay was performed as described previously.[8] One unit (U) of activity which hydrolyzes 1 μmol of the substrate per min at pH 5.3 and 37 °C was assumed to correspond to 86 pmol of PDMase.[7]

Determination of Kinetic Constants. The kinetic constants of PDMase for TpONph, NphOpT, and PhPONph were determined by the measurements of the initial velocity of 4-nitrophenol liberation at various substrate concentrations. The reaction mixture contained, in a total volume of 2.0 cm^3, various amount of a substrate, sodium acetate buffer, pH 5.3 (100 μmol) and an appropriate amount of PDMase. The mixture was incubated at 37 °C for 10 min, then 0.5 mol dm^{-3}, Na_2CO_3 (1.0 cm^3) was added to stop the reaction. The liberated 4-nitrophenol was determined by measurement of the absorbance at 400 nm of the resulting solution using molar absorbance 18500. At least five different amounts of the substrate were used to cover a substrate concentration range involving K_m for the substrate. A computer-aided determination of K_m and k_{cat} was carried out basically according to the method of Wilkinson.[9]

High Performance Liquid Chromatography (HPLC). A high-speed liquid chromatograph HLC-803A (Toyo Soda) equipped with a variable wavelength UV monitor was used. The column was IEX-540 DEAE (4× 150 mm) from the same company. The conditions for elution and detection varied depending on the experiment and will be described each time. The flow rate was fixed at 1.0 cm^3 min^{-1}.

Chemical Syntheses. *4-Nitrophenyl Phenylphosphonate*: The method described by Kelly *et al.*[2] was modified as follows. The solutions of phenylphosphonic dichloride and 4-nitrophenol in dry pyridine, each 16 cm^3 containing 41 mmol of either reagent, were added dropwise at approximately equal rates into 10 cm^3 of dry pyridine under stirring over 2 h. The temperature of the reaction mixture was maintained at 22—

30 °C by cooling in an ice bath. After the complete addition of the reagents, the mixture was stirred overnight at room temperature, then poured onto ice (100 g). After the ice thawed, the solution was adjusted to pH 1 with concd HCl (about 30 cm^3) and extracted three times with chloroform (each 70 cm^3). The combined chloroform layer was washed three times with 1 mol dm^{-3} HCl and twice with half-saturated aqueous NaCl. The chloroform solution was dehydrated with anhydrous Na_2SO_4, filtered and evaporated *in vacuo*. The residue was dissolved in acetone (15 cm^3). Concd aqueous ammonia was added to the solution until pH 7, then the solution was cooled in an ice bath for 1 h. The resulting precipitate of the ammonium salt of PhPONph was collected by centrifugation, washed extensively with cold acetone until no 4-nitrophenol was detected in the supernatant, and dried *in vacuo*. Yield 54%. Found: C, 48.79; H, 4.36; N, 9.27%. Calcd for $C_{12}H_{13}O_5N_2P$: C, 48.68; H, 4.43; N, 9.46%. UV_{max} (H_2O) 292 nm (ε 9800). ^1H NMR (D_2O) δ=8.67 (2H, d, J=9.0 Hz), 8.06—8.54 (5H, m), and 7.73 (2H, d, J=9.0 Hz).

O-(4-Nitrophenyl) Phenylphosphonothioate. Although Dudman and Benkovic[3] obtained the title compound by partial hydrolysis of the corresponding diester, we developed a new method for direct synthesis of the monoester. To phenylphosphonothioic dichloride (24 mmol) in 15 cm^3 of dry pyridine, 4-nitrophenol (24 mmol) in 12 cm^3 of dry pyridine was added dropwise under stirring over 2 h. The temperature of the reaction mixture was maintained at 2—10 °C by cooling in an ice bath. After the complete addition, the mixture was stirred overnight at room temperature, then poured onto ice (65 g). The product was extracted with chloroform (total 60 cm^3) as described in the synthesis of PhPONph. The extract was evaporated up and water (20 cm^3) was added to the residue. The mixture solidified on cooling. The resulting mass of the diester was triturated after addition of ethanol (20 cm^3) and filtered off. The filtrate was evaporated up and the residue was redissolved in water (20 cm^3). After a small amount of insoluble material was filtered off, 1 mol dm^{-3} cyclohexylammonium chloride (25 cm^3) was added to the solution. The precipitate formed on cooling was filtered, dried *in vacuo*, and recrystalized from hot acetonitrile. Yield 41%. Found: C, 54.52; H, 6.16; N, 7.40%. Calcd for $C_{18}H_{23}O_4N_2PS$: C, 54.81; H, 5.88; N, 7.10%. UV and ^1H NMR data agreed with those reported by Dudman and Benkovic.[3] The preparation, however, turned out to contain about 2% of PhPONph as described later.

Isolation of R_p Diastereomer of PhP(S)ONph. One of the diastereomers of chiral PhP(S)ONph was obtained by treatment of the synthetic racemate with snake venom phosphodiesterase. The reaction mixture contained, in a total volume of 12.1 cm^3, PhP(S)ONph (100 μmol), Tris–HCl buffer, pH 8.8 (500 μmol), $MgCl_2$ (10 μmol) and snake venom phosphodiesterase (0.28 U). The mixture was incubated at 37 °C for 25 h, then adjusted to pH 1 with concd HCl and extracted twice with chloroform (each 6 cm^3). The extract was charged on a column (2.2 cm × 14 cm) of silica gel preequilibrated with chloroform. After 4-nitrophenol was washed away with ethyl acetate (150 cm^3), the diastereomeric PhP(S)ONph was eluted with 1 : 1 mixture (50 cm^3) of ethyl acetate and methanol. The eluate was evaporated to dryness, taken up in water (2.0 cm^3), and adjusted to pH 7 with a dilute NaOH solution. The diastereomer thus obtained was not hydrolyzed by snake venom phosphodiesterase and showed CD ($[\theta]_{290\ nm}$ +630° $cm^2\ dmol^{-1}$).

Results

Hydrolysis of TpONph and NphOpT. PDMase hydrolyzed both TpONph and NphOpT, but in different ways. TpONph was degraded into thymidine, inorganic phosphate and 4-nitrophenol (Fig. 1A). A small amount of thymidine 3′-phosphate was detected, whereas 4-nitrophenyl phosphate was not. These results demonstrate that the two ester bonds in TpONph are hydrolyzed processively at the active site of PDMase. Presumably, 4-nitrophenol is the first leaving group, because thymidine 3′-phosphate, though little, was detected during the hydrolysis. On the contrary, NphOpT was cleaved first into 4-nitrophenol and

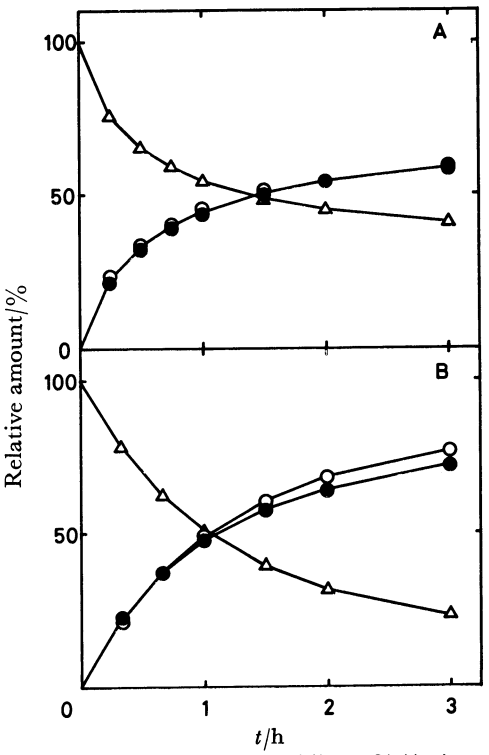

Fig. 1. Hydrolysis of thymidine 3′-(4-nitrophenylphosphate) (A) and thymidine 5′-(4-nitrophenylphosphate) (B) by PDMase.
The reaction mixture contained, in a total volume of 0.12 cm^3, TpONph (340 nmol) or NphOpT (220 nmol), sodium acetate buffer, pH 5.3 (15 μmol) and PDMase (116 mU). Incubation was at 37 °C. At the indicated times, portions (0.01 cm^3) of the mixture were withdrawn and analyzed by HPLC. The eluents were 0.25 and 0.125 mol dm^{-3} KH_2PO_4 for experiments A and B, respectively. Each compound was detected by absorbance at 290 nm and determined on the basis of peak height. Other conditions of HPLC were described in Experimental. The amount of each conpound is expressed in per cent to the sum of 4-nitrophenol and the remaining substrate. The retention times of the compounds were: (A) thymidine (●) 5.3 min, 4-nitrophenol (○) 11.2 min, TpONph (△) 13.1 min; (B) thymidine 5′-phosphate (●) 9.8 min, 4-nitrophenol (○) 11.3 min, NphOpT (△) 14.4 min. Besides the compounds described above, a small and nearly constant amount (less than 1%) of thymidine 3′-phosphate (retention time, 7.7 min) was detected in experiment A. A small and increasing amount (1.8% at 3 h) of thymidine (retention time, 5.3 min) was observed also in experiment B.

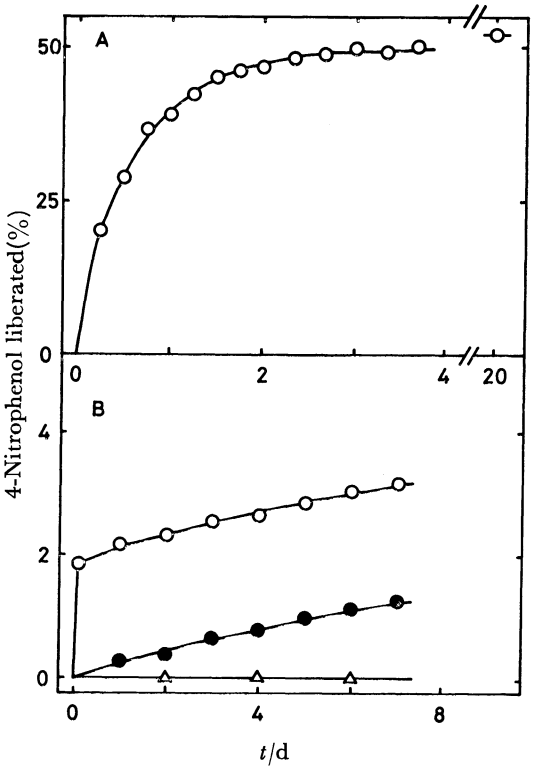

Fig. 2. Hydrolysis of *O*-(4-nitrophenyl) phenylphosphonothioate by snake venom phosphodiesterase (A) and PDMase (B). (A) The reaction mixture contained, in a total volume of 2.0 cm^3, PhP(S)ONph, racemate (2 μmol), Tris–HCl buffer, pH 8.8 (100 μmol), $MgCl_2$ (2 μmol) and snake venom phosphodiesterase (28 mU). Incubation was at 37 °C. At the indicated times, portions (0.05 cm^3) of the mixture were withdrawn and analyzed for 4-nitrophenol. (B) The reaction mixture contained, in a total volume of 1.0 cm^3, PhP(S)ONph, racemate (○) or R_p diastereomer (●) (3.5 μmol), sodium acetate buffer, pH 5.3 (50 μmol) and PDMase (3.9 U). Incubation was at 37 °C. At 1 h intervals, portions (0.1 cm^3) of the mixture were withdrawn and assayed for 4-nitrophenol. A control experiment (△) with the racemate was run in the absence of the enzyme under otherwise the same conditions.

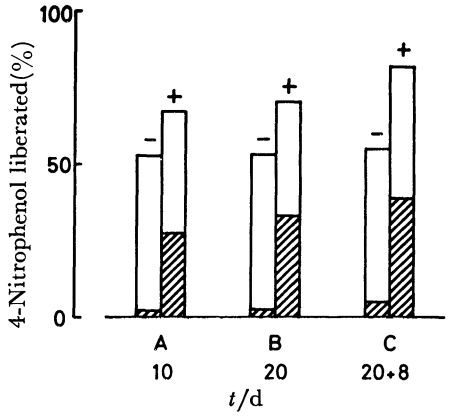

Fig. 3 Examination of the stereochemical course of hydrolysis of *O*-(4-nitrophenyl) phenylphosphonothioate by PDMase using snake venom phosphodiesterase. PhP(S)ONph, racemate (2.2 μmol) was incubated with sodium acetate buffer, pH 5.3 (50 μmol), NaN_3 as an antiseptic (1 mg) and PDMase (19 U) in a total volume of 1.0 cm^3 at 37 °C (first incubation). After 10 d (A) or 20 d (B) of the incubation, a portion (0.05 cm^3) of the mixture was withdrawn and assayed for 4-nitrophenol. Another portion (0.2 cm^3) of the mixture was taken and incubated with Tris–HCl buffer, pH 8.8 (40 μmol), $MgCl_2$ (0.4 μmol) and snake venom phosphodiesterase (12 mU) in a total volume of 0.4 cm^3 at 37°C (second incubation). After 3 h, a portion (0.1 cm^3) of the mixture was analyzed for 4-nitrophenol. The first incubation was renewed after 20 d: 0.2 cm^3 of the reaction mixture was mixed with 0.2 cm^3 of PDMase (7.5 U) solution in 10 mmol dm^{-3} sodium acetate buffer (pH 5.3), incubated at 37 °C for 8 more days (C), and analyzed as described above. The plus and minus signs on the histograms show the presence and absence, respectively, of PDMase during the first incubation. Hatched and blank areas in the histograms represent the percentage of 4-nitrophenol liberated by the first and second incubations, respectively.

thymidine 5′-phosphate, which was dephosphorylated very slowly (Fig. 1B). The kinetic constants for these substrates were determined to be: $K_m=(4.6\pm0.2)$ mmol dm^{-3} and $k_{cat}=(115\pm3)s^{-1}$ for TpONph; $K_m=(0.46\pm0.01)$ mmol dm^{-3} and $k_{cat}=(19.7\pm0.1)s^{-1}$ for NphOpT at pH 5.3 and 37 °C.

Hydrolysis of PhPONph and PhP(S)ONph. PhPONph was a good substrate for PDMase. The kinetic constants were $K_m=(1.4\pm0.3)$mmol dm^{-3} and $k_{cat}=(360\pm40)s^{-1}$ at pH 5.3 and 37 °C. The chiral thio analogue PhP(S)ONph synthesized by us was hydrolyzed by snake venom phosphodiesterase to an extent of 52.1% (Fig. 2A), confirming the observation of Dudman and Benkovic.[3] When this preparation was subjected to PDMase action, a portion (1.8%) was hydrolyzed rapidly but the rest was cleaved only very slowly (Fig. 2B). The R_p diastereomer was hydrolyzed by PDMase at almost the same rate as the racemate. The initial burst of 4-nitrophenol observed with the racemate but not with the R_p diastereomer may be due to contaminating PhPONph, which had resulted from desulfurylation of PhP(S)ONph probably during the isolation procedure in the synthesis.

The rate of 4-nitrophenol liberation from PhP(S)-ONph shown in Fig. 2B (25 pmol min^{-1} U^{-1}) corresponds to only 2×10^{-3}% of the rate which would be observed with PhPONph as the substrate under the same conditions. The low rate of hydrolysis is not due to an extremely high K_m, because the reduction of substrate concentration by a factor of 1/2 did not affect the rate appreciably (data not shown).

Although PhP(S)ONph (racemate) was hydrolyzed very slowly by PDMase, a considerable portion of the substrate could be hydrolyzed by a prolonged incubation with a large amount of the enzyme. When the remaining substrate was treated with snake venom phosphodiesterase, half its amount was always susceptible to the enzyme action (Fig. 3). These results indicate that neither diastereomer was concentrated in the remaining substrate. Therefore, PDMase hydrolyzed both dia-

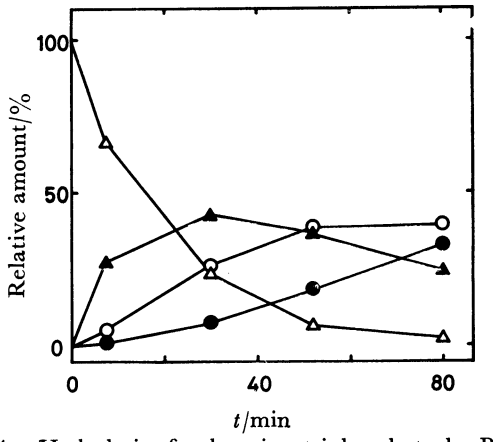

Fig. 4. Hydrolysis of adenosine triphosphate by PDMase. The reaction mixture contained, in a total volume of 0.21 cm^3, ATP (1.1 μmol), sodium acetate buffer, pH 5.3 (10 μmol) and PDMase (350 mU). Incubation was at 37°C. At the indicated times, portions (4 mm^3) of the mixture were withdrawn and analyzed by HPLC. The eluent was 0.5 mol dm^{-3} KH_2PO_4. Each compound was detected by absorbance at 260 nm and determined on the basis of peak area. Other conditions of HPLC were described in Experimental. The retention times of the compounds were: adenosine (●) 5.5 min, AMP (○) 8.9 min, ADP (▲) 13.9 min and ATP (△) 21.6 min.

stereomers at the same rate.

Hydrolysis of ATP. Figure 4 shows that ATP yielded adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine successively on incubation with PDMase. This indicates that the three phosphoryl groups in ATP is removed by PDMase in a sequential way from the terminal position.

Discussion

4-Nitrophenyl esters of thymidine 3′-phosphate and 5′-phosphate are currently used as key substrates for distinguishing phosphodiesterases between snake venom type and spleen type. Snake venom phosphodiesterase is active with NphOpT, whereas spleen phosphodiesterase is active with TpONph. The present study has shown that *Fusarium* PDMase acts on either of these substrates. Recently, Matsuzaki and Hashimoto[10] also reported that the acid phosphodiesterase of cultured tobacco cells was capable of hydrolyzing both NphOpT and TpONph. The dual activity may therefore be a feature common to acid phosphodiesterases of fungal or plant origin.

PDMase hydrolyzed the two chromogenic substrates in different ways. Processive hydrolysis occurred with TpONph as a substrate, whereas hydrolysis of NphOpT proceeded in a stepwise manner. The processive hydrolysis of a phosphodiester is the most prominent feature of PDMase. For example, bis(4-nitrophenyl) phosphate has been shown to be hydrolyzed into two moles of 4-nitrophenol and one mole of inorganic phosphate without appreciable release of the intermediary phosphomonoester, 4-nitrophenyl phosphate.[8] The same phenomenon was observed with TpONph. Presumably, stepwise hydrolysis of NphOpT resulted from the resistance of thymidine 5′-phosphate to PDMase action. The kinetic constants for these substrates were also different by an order of magnitude. Although the reason for such a big difference is not clear at present, it is worth noting that the k_{cat}/K_m value, a measure for goodness of a substrate, is of the same magnitude for these two substrates.

Kelly *et al.*[2] showed that phenylphosphonate esters are hydrolyzed by phosphodiesterase of snake venom type (5′-nucleotide phosphodiesterase according to their terminology), but not by spleen phosphodiesterase. Thus they proposed that phosphonate esters could serve as key substrates for distinguishing between the two types of phosphodiesterases. We have shown that PhPONph is a good substrate of PDMase. In fact, this compound has the highest k_{cat} (360 s^{-1}) among the substrates so far examined.

Dudman and Benkovic[3] demonstrated that the chiral thio analogue of PhPONph, PhP(S)ONph, is hydrolyzed by snake venom phosphodiesterase in a stereospecific manner. They claimed that the thio analogue is hydrolyzed by the same pattern of enzymes which catalyze hydrolysis of PhPONph. Further studies on the stereochemical course of snake venom phosphodiesterase strongly suggest that the S_p diastereomer of PhP(S)ONph is hydrolyzed specifically by the enzyme.[4,11,12] We have shown that the thio analogue is a poor substrate of PDMase, although the parent phosphonate ester is readily hydrolyzed by the enzyme. The rate of hydrolysis of PhP(S)ONph by PDMase was only 2×10^{-3}% of that of PhPONph. Both diastereomers of PhP(S)ONph seems to be hydrolyzed by the enzyme at almost equal rates. These results are in sharp contrast to the observation made with snake venom phosphodiesterase.

As for the mode of hydrolysis of ATP, PDMase also differs from snake venom phosphodiesterase. PDMase removes the three phosphoryl groups in ATP sequentially from the terminal position, whereas snake venom phosphodiesterase splits ATP into AMP and inorganic pyrophosphate.[4]

Precise comparison is now feasible between *Fusarium* PDMase and snake venom phosphodiesterase on the basis of our present and previous studies. PDMase, though unique in its wide substrate specificity, has certain resemblances to snake venom phosphodiesterase. (1) It yields 5′-nucleotide on acting diribonucleoside monophosphate.[5] (2) It hydrolyzes PhPONph. However, PDMase clearly differs from snake venom phosphodiesterase, to say nothing of pH optima and metal ion requirements, in the following points. (1) It hydrolyzes TpONph in addition to NphOpT. (2) It hydrolyzes chiral PhP(S)ONph only very slowly in a stereo-nonspecific manner. (3) It removes the three phosphoryl groups in ATP sequentially from the terminal position.

As a conclusion, the present study has made the uniqueness of *Fusarium* PDMase clearer. It does not seem to fit any entry of the current phosphohydrolase classification. We propose that a new entry be necessary for this unique enzyme, most appropriately among

EC 3.1.4 phosphoric diester hydrolases.

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