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PDM Phosphatase Cleaves the P—O Bond of *p*-Nitrophenyl Phenylphosphonate

(phosphatase／P—O bond cleavage／enzyme mechanism)

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***p*-Nitrophenyl phenylphosphonate was hydrolyzed by PDM phosphatase in water labeled with ^{18}O. Mass spectrometric analysis of the products showed that phenylphosphonic acid contained ^{18}O but *p*-nitrophenol did not. This indicates that PDM phosphatase cleaves the P—O bond of the substrate.**

Introduction

PDM phosphatase is a unique phosphohydrolase (phosphoric acid hydrolyzing enzyme) isolated from the culture filtrate of a phytopathogenic fungus, *Fusarium moniliforme* (1). This enzyme is unique because it hydrolyses both phosphomonoesters and phosphodiesters. In the enzyme classification, phosphohydrolases are divided into two major groups : phosphomonoesterases and phosphodiesterases. PDM phosphatase is an exception to this rule. Therefore, it is of interest to compare PDM phosphatase with other phosphohydrolases with respect to basic enzymatic properties and to find out factors which make PDM phosphatase unique.

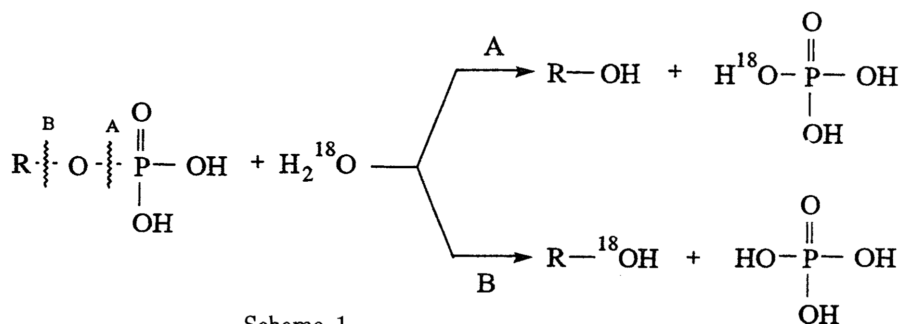
One of the fundamental problems in the mechanism of phosphohydrolases is whether the hydrolysis proceeds via P—O or R—O, hence C—O cleavage. The two possibilities can be distinguished by use of $[^{18}O]H_2O$. As shown in Scheme 1, if P—O cleavage takes place, phosphoric acid will be labeled with ^{18}O (path A), whereas the alcohol will be labeled in case of C—O cleavage (path B).

Since the classic demonstration of P—O cleavage by Stein and Koshland with an alkaline phosphatase (2), the concept has been widely accepted so that usually P—O cleavage is supposed without experimental verification in the mechanism of any phosphohydrolase.

In view of the uniqueness of PDM phosphatase, we thought that it might be worth verifying whether P—O cleavage actually occurs also with this enzyme.

Materials and Methods

PDM phosphatase with a specific activity of 37.7 was obtained from the dried powder of



Scheme 1

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This paper is respectfully dedicated to the memory of the late Professor Masami Kurokawa.

culture filtrate of *Fusarium moniliforme* through five step purification. The method is considerably different from that published earlier (3), but the detail will be described elsewhere. Ammonium salt of *p*-nitrophenyl phenylphosphonate was synthesized as described previously (4). [^{18}O]H_2O with an ^{18}O content of 72.9 atom% was purchased from Prochem B.O.C. Limited.

High performance liquid chromatography (HPLC) was carried out with a Shimadzu liquid chromatograph LC-6A using a Cosmosil packed column 5C18 (4.6 i.d.×250mm). Elution was performed at a flow rate of 1.0 ml/min by a linear gradient from 1% aqueous trifluoroacetic acid to acetonitrile containing 1% trifluoroacetic acid in 25 min. Detection was by absorbance at 260 nm. *p*-Nitrophenyl phenylphosphonate, *p*-nitrophenol and phenylphosphonic acid could be separated by this system (data not shown).

Mass spectrometry was performed with a JEOL JMS-AX505HA mass spectrometer. Samples in glycerol matrices were bombarded by a beam of fast atoms and the resulted anions were analyzed. The anion mode was selected, because both *p*-nitrophenol and phenylphosphonic acid were expected to lose a proton more easily than to get one.

Results and Discussion

In this study, *p*-nitrophenyl phenylphosphonate was chosen as the substrate from the following reasons. First, both of its enzymatic cleavage products are expected to be easily analyzed for ^{18}O contents by mass spectrometry. If a phosphomonoester were used, one of the products, phosphoric acid, would not be subjected to mass spectrometric analysis without some derivatization. Second, although this substrate is a phosphonic acid monoester, it can be regarded as a phosphodiester analogue. Therefore, the present study will provide information on the enzymatic mechanism of phosphodiesterase action of PDM phosphatase. So far, no investigation has been made on any phosphodiesterase with regard to the bond cleaved in the course of hydrolysis.

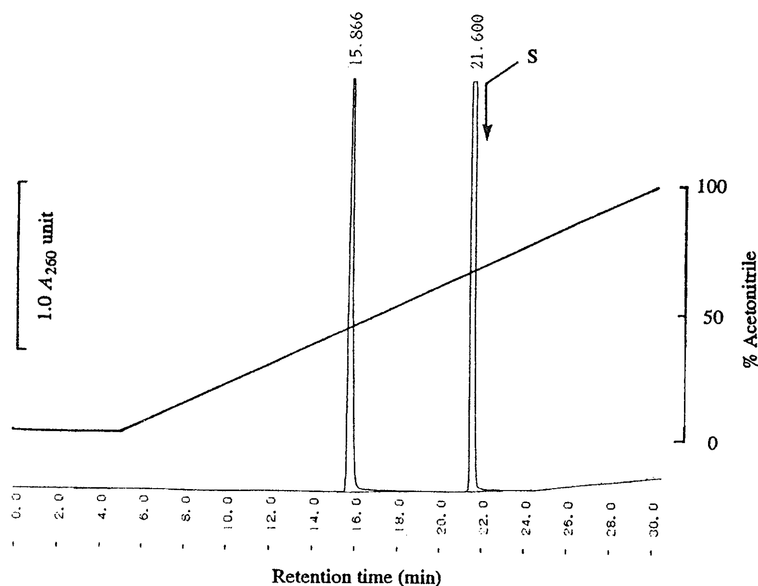


Fig. 1. HPLC elution profile of the reaction products of the hydrolysis of *p*-nitrophenyl phenylphosphonate by PDM phosphatase. Experimental details are described in the text. The peak at 15.9 min is phenylphosphonic acid and that at 21.6 min is *p*-nitrophenol. The arrow S indicates the elution position of the substrate (22.0 min).

The reaction mixture contained 5 μmol of *p*-nitrophenyl phenylphosphonate, 5 μmol of sodium acetate buffer (pH 5.3) and 0.52 unit of PDM phosphatase in 0.4 ml of $[^{18}O]H_2O$ with 71.8 atom% of ^{18}O. After the mixture was incubated for 1 h at 37℃, it was subjected to HPLC separation in 0.1-ml portions. During the incubation, the substrate was completely degraded into *p*-nitrophenol and phenylphosphonic acid as shown in Fig. 1.

The peaks of the products were separately pooled, then the pools were evaporated *in vacuo* and subjected to mass spectrometric analysis. The mass spectra shown in Fig. 2 clearly indicate that *p*-nitrophenol contained no ^{18}O. On the contrary, 63.7% of phenylphosphonic acid possessed a mass number two units higher than that expected from its relative molecular mass, showing incorporation of one oxygen atom into phenylphosphonic acid from the medium water.

This indicates that the P−O cleavage took place during the hydrolysis as shown in Scheme 2.

Presumably, in the course of action of PDM phosphatase, a water molecule attacks the phosphorus atom nucleophilically either directly or after formation of an intermediate covalently bound to an active site residue of the enzyme. Thus, PDM phosphatase, though unique among

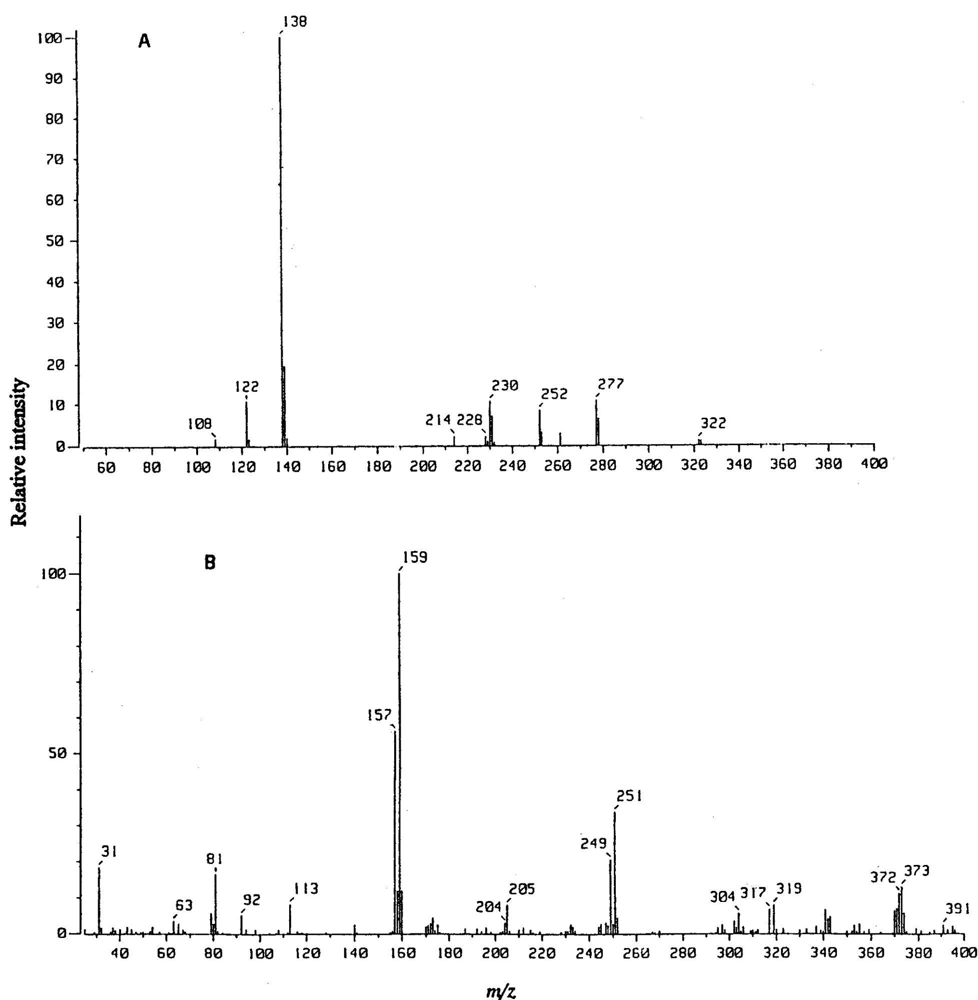
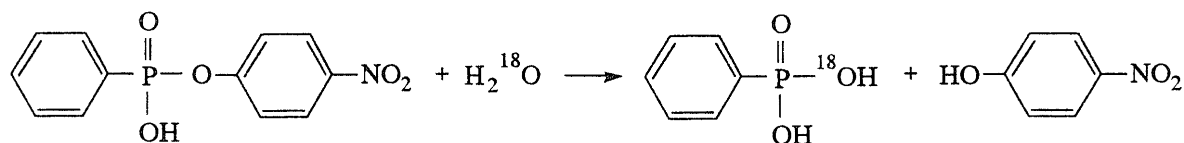


Fig. 2. Mass spectra for the *p*-nitrophenol (M_r=139)(A) and the phenylphosphonic acid (M_r=158)(B) obtained as described in the text. The $(M-H)^-$ ions were detected for both compounds. The peaks of glycerol (M_r=92) adducts were also observed at considerable intensities with phenylphosphonic acid.



Scheme 2

phosphohydrolases, follows the general mechanism for enzymes of this class as far as the cleavage site concerns. A search for the residues which assist the nucleophilic attack of a water molecule will be the subject of our future investigation.

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