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Cloning and Sequencing of cDNA and Genomic DNA Encoding PDM Phosphatase of *Fusarium moniliforme*

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PDM phosphatase was purified approximately 500-fold through six steps from the extract of dried powder of the culture filtrate of *Fusarium moniliforme*. The purified preparation appeared homogeneous on SDS-PAGE although the protein band was broad. Amino acid sequence information was collected on tryptic peptides from this preparation. cDNA cloning was carried out based on the information. A full-length cDNA was obtained and sequenced. The sequence had an open reading frame of 651 amino acid residues with a molecular mass of 69,988 Da. Cloning and sequencing of the genomic DNA corresponding to the cDNA was also conducted. The deduced amino acid sequence could account for many but not all of the tryptic peptides, suggesting presence of contaminant protein(s). SDS-PAGE analysis after chemical deglycosylation showed two proteins with molecular masses of 58 and 68 kDa. This implied that the 58 kDa protein had been copurified with PDM phosphatase. Homology search showed that PDM phosphatase belongs to the purple acid phosphatase family, which is widely distributed in the biosphere. Sequence data of fungal purple acid phosphatases were collected from the database. Processing of the data revealed presence of two types, whose evolutionary relationships were discussed.

Key words: cDNA cloning, *Fusarium moniliforme*, genomic DNA cloning, PDM phosphatase, purple acid phosphatase.

Abbreviations: ORF, open reading frame; Pase, phosphatase; PAP, purple acid phosphatase; SSC, saline-sodium citrate (20× SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0); TFA, trifluoroacetic acid.

In 1971, Yoshida and Tamiya reported purification and properties of an acid phosphatase (Pase) from the culture filtrate of a phytopathogenic fungus, *Fusarium moniliforme* (*1*). Further studies by us revealed its unique substrate specificity and led us to propose a new name, phosphodiesterase-phosphomonoesterase (*2*), which was later altered to PDM Pase for the sake of simplicity (*3*). It hydrolyzes not only phosphomonoesters but also a synthetic phosphodiester in a processive manner (*2*), pyrophosphates (*1*), 2′,3′-cyclic nucleotides (*4*), diribonucleoside monophosphates (*5*), and homopolyribonucleotides (*3*). Structural basis for such wide specificity is of great interest, since no other Pase with similar properties has so far been reported. However, structural studies have been hampered by its high molecular mass (*ca.* 100 kDa) (*6*), high carbohydrate content (approximately 40 weight % of protein) (*7*), and presence of multiple forms with different isoelectric points (*7*). cDNA cloning will naturally be an approach of choice in such a case. With this aim in mind, we first developed a new method of purification. Based on the amino acid sequence information collected on the purified preparation, we could successfully clone the cDNA and genomic DNA encoding PDM Pase. The enzyme turned out to belong to the purple acid phosphatase (PAP) family, which is widely distributed in plants, mammals, fungi, and bacteria (*8*). The study, however, brought us an unexpected incidental finding: the purified PDM Pase preparation contained a substantial amount of a single species of contaminant protein. Here we describe the overall process for the cloning and how we reached the finding.

MATERIALS AND METHODS

Materials, Enzyme Assay, and Electrophoresis—The following enzymes were purchased from Takara: restriction endonucleases, bacterial alkaline phosphatase, T4 polynucleotide kinase, T4 DNA ligase, and Taq DNA polymerase. Trypsin was obtained from Worthington. Phenyl-Sepharose CL-4B, Sepharose CL-6B, PBE-94, and Polybuffer 74 were from Pharmacia. SP-Toyopearl 650M was from Tosoh. PDM Pase activity was assayed as described previously (*2*) using *p*-nitrophenyl phosphate as a substrate. SDS-PAGE was run according to Weber and Osborn (*9*). Native PAGE and activity staining were carried out as described previously (*7*). Two-dimensional PAGE was conducted essentially according to Görg *et al.* (*10*). An Immobiline dry strip pH gradient 3–10

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(Amersham) was used after swelling for the first dimension, and a 12% polyacrylamide slab gel was used for the second dimension.

HF Treatment of the Purified Pase Preparation—This was carried out according to Mort and Lampart (*11*). The lyophilized protein sample (1.0 mg) was extensively dried over P_2O_5 and suspended in 0.5 ml of anisole. To the suspension was added 5 ml of anhydrous HF and the mixture was stirred at 0°C for 1 h. After the HF was evaporated *in vacuo*, the residue was extracted with three 0.5-ml aliquots of 33% acetic acid. The combined extract was treated three times with 1 ml each of ether for elimination of anisole, then gel-filtrated through Sephadex G-25 equilibrated with 0.1 M acetic acid. The protein fraction was lyophilized.

Determination of Amino Acid and Nucleotide Sequences—Peptides were sequenced with a protein sequencer model 477A (Applied Biosystems). DNA samples were sequenced on both strands with an ABI Prism 310 Genetic Analyzer using ABI Prism BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) with appropriate oligonucleotide primers which had been synthesized and supplied on order by Pharmacia.

Trypsin Digestion of the Purified Pase Preparation and Separation of the Tryptic Peptides—The sample protein was desalted, reduced and carboxymethylated according to Crestfield *et al.* (*12*). The resulting product (2 mg) was dissolved in 1 ml of 50 mM ammonium bicarbonate and digested at 37°C for 6 h with 20 μg of trypsin which had been treated with *N*-tosyl-L-phenylalanyl chrolomethyl ketone. The reaction mixture was lyophilized, dissolved in 600 μl of 0.1 M acetic acid and subjected to reversed phase HPLC in 40 μl portions. Conditions for HPLC were as follows: column, Cosmosil 5C18 (4.6 × 250 mm, Nacalai) annexed with a precolumn (4.6 × 50 mm); elution program, 0.1% trifluoroacetic acid (TFA) for the initial 5 min, a linear gradient from 0 to 42% CH_3CN in 0.1% TFA for the next 40 min, and a linear gradient from 42 to 70% CH_3CN in 0.1% TFA for the final 5 min; flow rate, 1.0 ml/min; detection, absorbance at 230 nm.

Construction of cDNA and Genomic DNA Libraries of F. moniliforme—Culture of *F. moniliforme* and isolation of the total RNA were carried out as described previously (*13*). Poly(A)$^+$ RNA or mRNA was isolated from the total RNA using BioMag (PerSeptive Diagnostics). A double stranded cDNA was prepared from the mRNA using cDNA synthesis kit (Amersham). The cDNA was then ligated to the following adaptor purchased from Pharmacia using T4 DNA ligase:

5′-AATTCGCGGCCGCT-3′
3′- GCGCCGGCGAp-5′

The adaptor-ligated cDNA was kinated and ligated to λZAP II (Stratagene). The construct was then packaged into λphage using Gigapak III Gold (Stratagene) yielding a cDNA library with a titer of 1.4×10^6 plaques forming units/ml. The DNA of *F. moniliforme* was isolated from mycelia by the method of Murray and Thompson (*14*). After cleavage with *Eco*RI, the digested DNA was directly ligated to λZAP II and packaged. A genomic library with a titer of 7.0×10^6 plaques forming units/ml was obtained. Another genomic library with a titer of 5.0×10^7 plaques forming units/ml was obtained by digestion of the DNA with *Eco*RV, dephosphorylation, ligation to the above mentioned adaptor, and the treatment as described above. Inserts of the constructed phages could be excised and subcloned into pBluescript phagemids with the use of a helper phage according to the manual provided by the packaging kit supplier.

PCR Reactions—These were carried out using reagents provided by Takara. The reaction mixture contained 10 μl of 10 × PCR buffer, 8 μl of dNTP mix, approximately 50 μg of a DNA sample, each 1 μl of primer solutions (150 μM for redundant primers or 15 μM for unique primers) and 0.5 μl of Taq DNA polymerase in a total volume of 100 μl. The PCR conditions were as follows: (i) initial 3 min denaturation at 94°C, (ii) 30 cycles of 1 min denaturation at 93°C, 1 min annealing at 55°C and 2 min elongation at 72°C, and (iii) final 5 min incubation at 72°C.

Hybridization—Phage plaques were blotted onto Hybond-N$^+$ nylon membranes (Amersham) according to the standard procedure (*15*). Then, the membranes were hybridized with a digoxigenin labeled probe at 42°C overnight in 5× saline–sodium citrate (SSC) containing 50% formamide, 2% blocking solution, 0.1% lauryl sarcosine and 0.02% SDS. The blots were subsequently washed twice in 2× SSC, 0.1% SDS for 5 min at room temperature, then twice in 0.1× SSC, 0.1% SDS for 15 min at 68°C. The washed membranes were subjected to immunochemical detection. Digoxigenin labeling and the detection were performed using kits from Boehringer.

Sequence Data Search and Analysis—These were carried out at GenomeNet Server (Kyoto Center). Homology search was conducted using FASTA. Multiple alignment of amino acid sequences and construction of a phylogenetic tree thereof were made using Clastal W.

RESULTS

Purification of PDM Pase—1) Ethanol precipitation: The crude extract of dried powder of the culture filtrate of *F. moniliforme* was fractionated with ethanol as described previously (*6*). In short, the powder (100 g) was extracted twice with each 300 ml of 10 mM acetate buffer, pH 5.3 (buffer A). Then, the crude extract was added with 0.5 volume of ethanol on ice and the resulting precipitate was collected by centrifugation at 11,000 × *g* for 20 min at 4°C. The pellet was extracted with 100 ml and again 50 ml of buffer A. This procedure was repeated a number of times and the obtained enzyme solutions were pooled and stored frozen at –20°C until use. Purification in this report was started from a total of 7.73 liters of the stock which had been purified approximately five fold over the crude extract. Each of the following steps was repeated several times. Column chromatographies were carried out at 4°C except otherwise stated. Here we describe a typical experiment for each step.

2) Ammonium sulfate fractionation: Precipitate which appeared on thawing the stock was centrifuged off. To the enzyme solution (248 ml), ammonium sulfate (139 g) was added to 80% saturation. The mixture was left on ice for 1 h and the resulting precipitate was centrifuged off. PDM Pase remained soluble even in this high concentration of the salt.

3) Phenyl-Sepharose CL-4B hydrophobic chromatography: This was carried out at room temperature. The supernatant obtained above was loaded on a column (2.8 × 36 cm)

of Phenyl-Sepharose CL-4B which had been equilibrated with 80% saturated ammonium sulfate in buffer A. Elution was conducted at a flow rate of 1 ml/min by a decreasing linear gradient of ammonium sulfate from 80 to 0% saturation. The enzyme was eluted as a symmetric single peak at approximately 45% saturation. Active fractions were pooled and concentrated with a Labomodule (Asahi Chemical Industry Co.).

4) SP-Toyopearl 650M Ion exchange chromatography: The enzyme solution was further concentrated by ultrafiltration using PM-3 membrane (Amicon), then desalted on a Sephadex G-25 column equilibrated with 10 mM acetate buffer, pH 4.6 (buffer B). The enzyme solution (202 ml) was loaded onto a column (2.6 × 91 cm) of SP-Toyopearl 650M which had been equilibrated with buffer B. After the flow-through components had been washed away, the column was developed with buffer B containing 0.2 M NaCl at a flow rate of 2.4 ml/min. A sharp peak of the enzyme activity appeared. Active fractions were pooled and concentrated by the ultrafiltration.

5) Sepharose 6B Gel Filtration: The enzyme solution (21 ml) was loaded onto a column (2.6 × 88 cm) of Sepharose 6B equilibrated with buffer A containing 0.1 M NaCl. Elution was continued with the same solution at a flow rate of 0.84 ml/min. A symmetric activity peak roughly overlapping a major protein peak emerged at V_e/V_t = 0.84. Active fractions were pooled and concentrated by the ultrafiltration.

6) Chromatofocusing: The enzyme solution (20 ml) was passed through a Sephadex G-25 column equilibrated with 10% Polybuffer 74 (pH4.4). The active fractions separated from NaCl were pooled. The solution (46 ml) was loaded onto a PBE 94 column (1.5 × 56 cm) equilibrated with 25 mM imidazole–HCl buffer (pH 7.4). Elution was carried out with 10% Polybuffer 74 (pH 4.4) at a flow rate of 1.8 ml/min and 10-ml fractions were collected. The elution profile is shown in Fig. 1. Two major activity peaks coincident with those of protein were observed. Fractions I and II were brought to 80% saturation with solid ammonium sulfate. Then, the enzyme was adsorbed to a small column (1.5 × 21 cm) of Phenyl-Sepharose CL-4B equilibrated with buffer A containing 80% saturated ammonium sulfate. After Polybuffer detectable by A_{230} had been washed away by the same solution, the enzyme was recovered in a small volume by elution with buffer A.

Table 1 summarizes the purification procedure. Since the starting stock solution had been purified approximately five fold over the crude extract, an over-all purification of more than 500-fold was attained. Inspection of purity of the final preparation by SDS-PAGE or native PAGE gave essentially the same results as reported previously (7). Namely, each of fractions I and II gave a broad single band of the same mobility on SDS-PAGE (molecular mass 71–95 kDa). On native PAGE, each of them appeared as a broad protein band which was also detectable by activity staining. Fraction I moved slightly faster toward the anode than fraction II (data not shown). As discussed previously (7), band broadening might presumably be due to different degree of glycosylation. Therefore, we judged that fraction I at least was sufficiently pure.

Collection of Amino Acid Sequence Information on the Purified Preparation—Fraction I protein was reduced, carboxymethylated and digested with trypsin. The resulting

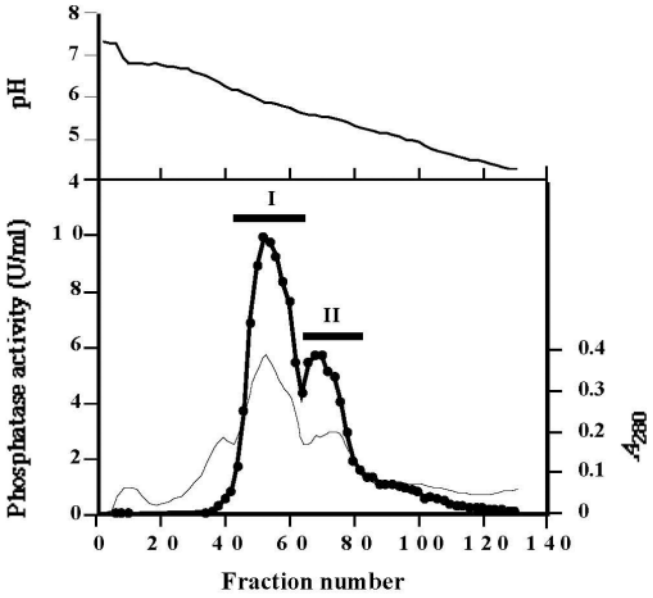

Fig. 1. **Elution profile of the chromatofocusing.** Experimental details are described in "MATERIALS AND METHODS." Enzyme fractions I and II were pooled as indicated by bars. The pH gradient is shown in the upper panel. Continuous line, A_{280}; filled circle, Pase activity.

tryptic peptides were separated by HPLC and fractions were pooled as indicated in Fig. 2. Purity of each fraction was checked by the same HPLC and purified further by rechromatography when necessary. Peptides which appeared homogeneous were sequenced. Finally, sequence information was obtained for a total of nearly 200 residues as shown in Table 2.

Cloning and Sequencing of a cDNA Encoding PDM Pase—Based on the information obtained above, we designed several sense and antisense primers and performed PCR reactions using the mRNA as a template in order to obtain a long probe. Combination of a sense primer GARCARTAYCARTGG (Ps1) and an antisense primer TCRTACCARTGNACRTGNCC (Pa6) gave an approximately 250-bp long PCR fragment. Ps1 corresponded to a part of peptide T20-5° (EQYQW). Pa6 was complementary to the sequence corresponding to a part of T22-2 (GHVHWYE). The PCR fragment was isolated, subcloned to pT7 blue T vector (Novagen) and sequenced. The translated amino acid sequence contained peptide T14-2°a besides the originally selected peptides at both ends, proving authenticity of the fragment. Using this fragment as a probe, we screened the cDNA library and obtained three positive clones from 6 × 10^4 phages. The inserts of these clones were excised into pBluescript phagemids and the resulting subclones were named pBPDc1–pBPDc3. Their cDNA inserts were analyzed for size after digestion with *Eco*RI. All of them gave fragments of the same size (2.2 kbp). Terminal sequence analysis showed that the cDNAs had the same sequences at both ends except for the length of poly(A) tract. Therefore, pBPDc2 was arbitrary chosen and its insert was completely sequenced on both strands. The sequence was 2,161-bp long and had an open reading frame (ORF) of 651 amino acid residues with a molecular mass of 69,988 Da (Fig. 3). The amino acid sequence began

Table 1. **Summary of the purification.**

Step	A_{280} unit[a] (kU)	Phosphatase activity (kU)	Specific activity[b]	Purification (fold)	Yield (%)
1) Ethanol precipitation	281	57.7	0.205	1	100
2) Ammonium sulfate fractionation	194	57.7	0.297	1.45	100
3) Phenyl-Sepharose CL-4B hydrophobic chromatography	35.5	48.4	1.36	6.63	83.4
4) SP-Toyopearl 650M ion exchange chromatography	2.59	14.7	5.68	27.7	25.5
5) Sepharose 6B gel filtration	1.13	17.2	15.2	74.1	29.8
6) Chromatofocusing					
Fraction I	0.254	7.09	27.9	136	12.3
Fraction II	0.112	2.69	24.0	117	4.7

[a]One unit was defined as that amount which was contained in 1.0 ml of a solution with A_{280} = 1.0. [b]Phospatase activity (U)/A_{280} U.

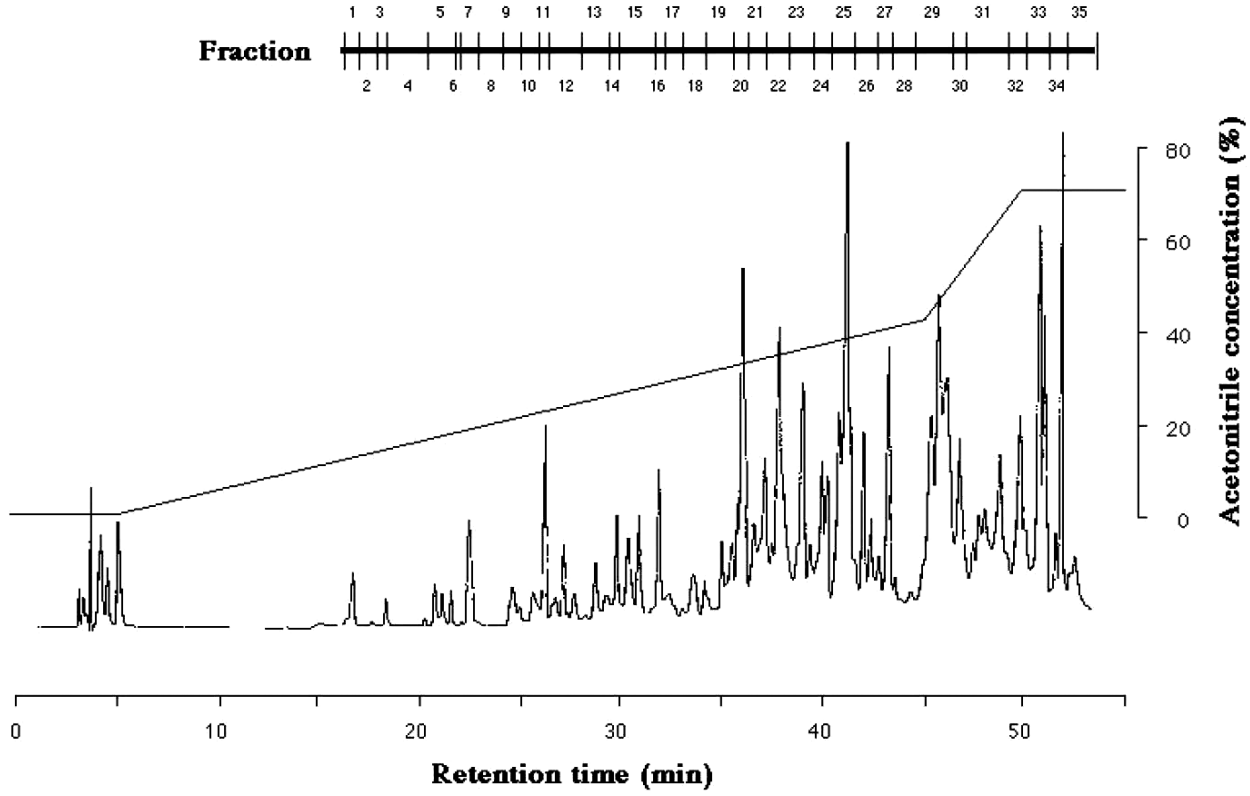

Fig. 2. **Reverse phase HPLC separation of tryptic peptides from the purified PDM Pase preparation.** Experimental details are described in "MATERIALS AND METHODS." Fractions were pooled as indicated on top of the figure.

with a Met residue followed by a likely pre-sequence, indicating that a full-length cDNA was obtained. Homology search revealed four closely related enzymes of the purple acid phosphatase (PAP) family from filamentous fungi of *Aspergillus* genus (Fig. 4).

Reinvestigation of Purity of the Purified Preparation of PDM Pase—Puzzlingly, however, the translated sequence could account for only about half of the sequenced tryptic peptides (Table 2). The most straightforward interpretation of this result was the following: the purified PDM Pase preparation contained a substantial amount of some other protein(s) despite 500-fold purification. Therefore, we checked its purity by two-dimensional electrophoresis and found a skew spot extending in a region, pI 5–6 and molecular mass 65–100 kDa (data not shown). As suggested previously (7), extension of the spot might have been caused by different degrees of glycosylation. Therefore, we deglycosylated the purified preparation with HF and analyzed the product by SDS-PAGE. As shown in Fig. 5, two bands with nearly equal intensities were observed at molecular masses of 58 and 68 kDa. The latter mass was close to that of PDM Pase deduced from the cDNA. This indicated that the purified preparation contained another glycoprotein with a protein molecular mass of 58 kDa and that its quantity was comparable to that of PDM Pase, the original target of the purification. In other words, this protein had been copurified with PDM Pase throughout the purification procedure.

Cloning and Sequencing of Genomic DNA Corresponding to the cDNA—In order to get further insight into the gene structure, we undertook the genomic DNA cloning. A new probe for screening was synthesized by PCR

Table 2. **Catalog of the tryptic peptides derived from the purified PDM Pase preparation.**

Peptides	Sequence	Presence[a]
T5-1	YLKK	–
T5-3	YPQDK	–
T5-4	GEVANQGSPR	+
T7-1a	YLK	–
T7-1b	GFQK	–
T7-2	YLNK	+
T7-4	ATGSTTTYDR	+
T12-1°-3′	VVDEKYPYK	+
T12-1°-4′	ATGSTTTYDRTPPCSAVK	+
T14-2°a	YQVNLR	+
T14-2°b	XFTAFQNR	+
T15-4a	LQPMGHNGTLDSGSVINNNT—	+
T15-4b	SDTSLAPAQTDVLK	–
T15-5	TYYYQIPAAXGTTK	+
T18-2	AXETYVTDAGPFGAVHGSY—	+
T18-3	FVVXGSAIPEVK	–
T20-4°	LSEEGIVGQWGEER	–
T20-5°	NYEQYQWLAK	+
T22-2	NNVDVYIAGHVHWYER	+
T24-2a	NFINTFELVK	–
T24-2b	QFYVPFHIDYNGGR	–
T25-3	GLTWYEVQLAGHELPGYSAGSGYR	–
T26-4	ALNAPTDVTWYQCKPNAVFGFGNP—	–
T27	LVEPPAVKPGSSNPSNNVNVISTSYTPGGI—	+
T29-1	KLYFWFFPSTNPK	–
T32-2/T33-3	SGGVGNFWYSFDYGLAHFVSINTETDYANSPAKPF—	+
T32-5	GATTYITGESYAGYYVPYIADAFITANDDD—	–
T32-7/T33-6	LGGVAINDPIIGDGTLQQQAVIYPYIEYW—	+
T33-4/T34-3	GGDMSVLYESNWDLWQQXLN—	+

[a] + and – indicate presence and absence, respectively, of that peptide in the amino acid sequence of PDM Pase deduced from the cDNA.

using the cDNA as a template and a combination of sense primer 53–72 (sequence number of the cDNA) and antisense primer 2005–1986. The PCR fragment covered the entire ORF region. Screening of the genomic library was carried out using this probe labeled with digoxigenin. First, we searched the genomic library constructed with the *Eco*RI digested DNA but failed to get any positive clone. Southern blot analysis of the digest showed that the *Eco*RI fragment containing the PDM Pase gene was on top of DNA smear in agarose gel electrophoresis (*ca.* 20 kbp, data not shown). Presumably, it was too long to be cloned into λZAP II. Therefore, we had to construct a new genomic library by digestion of the DNA with a blunt-end producing restriction endonuclease and ligation of the resulting fragments to λZAP II through the intermediary of the adaptor used for construction of the cDNA library. Preliminary experiments showed that *Eco*RV was suitable for this purpose: it produced a single 4–5 kbp positive fragment. A genetic library with a titer of 5×10^7 plaque forming units/ml was constructed. Screening of this library yielded two positive clones from 5×10^4 phages. The inserts of these clones were excised and the resulting phagemids were named pBPDg1 and pBPDg2. Their inserts were analyzed for size and terminal sequences. Both of them were shown to harbor the same 4.4 kbp fragment. Therefore, pBPDg2 was arbitrary chosen and its insert was completely sequenced on both strands, yielding 4,364-bp long sequence. The construction of the gene for PDM Pase is schematically shown in Fig. 6. It consists of three exons separated by relatively short (78 and 56 bp) introns. The sequences of the gene and the cDNA were perfectly consistent in their common regions.

DISCUSSION

We revised the previously reported method of purification of PDM Pase (*6*). The aim of the revision was to establish a method suitable for large-scale preparation in order to promote studies on this unique enzyme in various aspects. The previous method utilized chromatographies on hydroxyapatite, P-cellulose and DEAE-cellulose yielding a mixture of multiple forms, which were separated by isoelectric focusing. Although the method was effective, its scale-up did not work as expected. Especially, the hydroxyapatite chromatography, when scaled-up, could not reproduce the results obtained in the small-scale experiment. The finding that PDM Pase remains soluble in ammonium sulfate solution as high as 80% saturation led us to an idea of conducting hydrophobic chromatography. This method as described here turned out to have various advantages. First, under high concentration of ammonium sulfate, the enzyme was quite stable so that the chromatography could be carried out with a high yield at room temperature. Second, brown-colored material, which was present abundantly in the starting material and was the major obstacle for purification, flowed through the column, thus its large

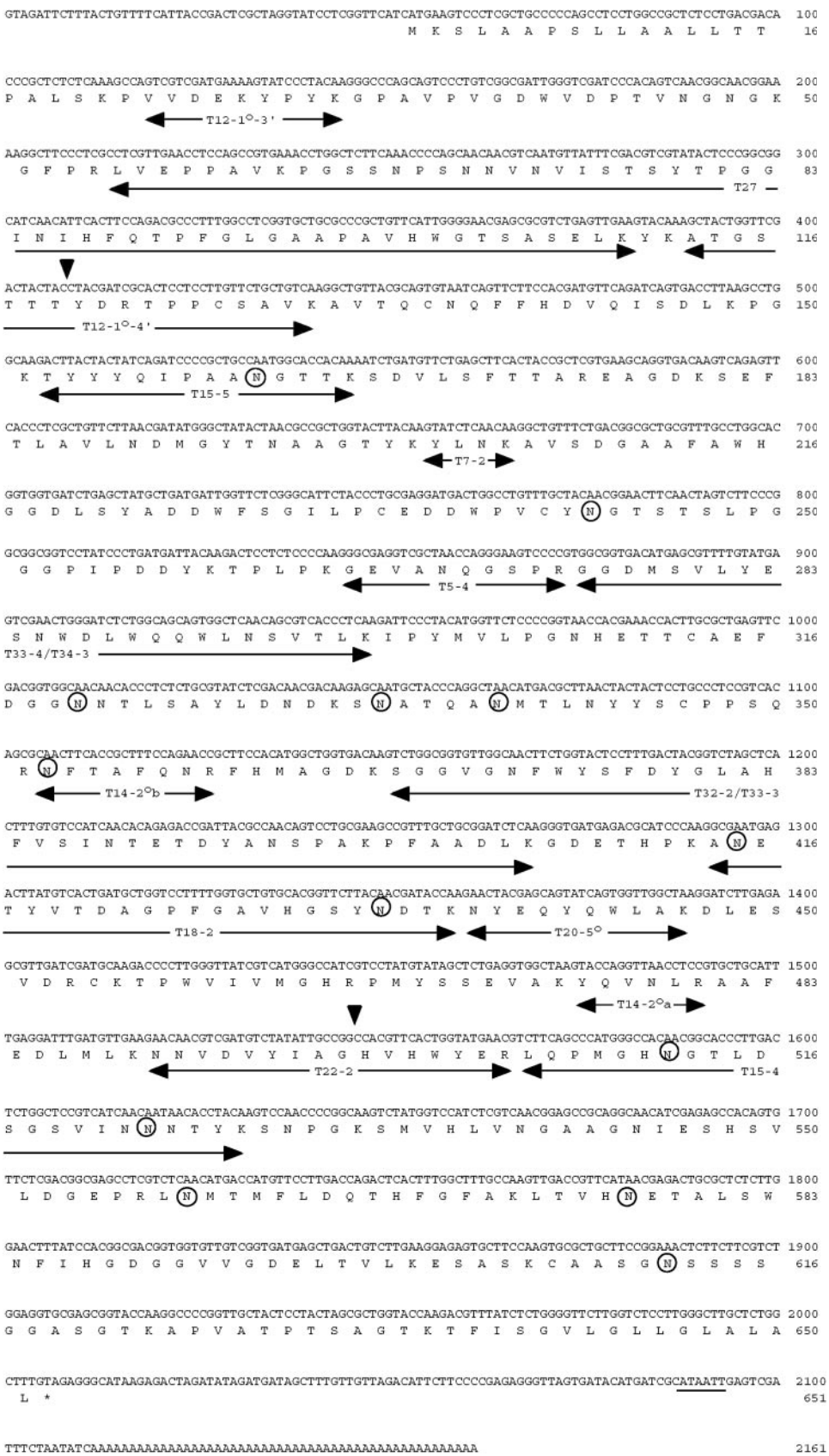

Fig. 3. **Nucleotide sequence of cDNA encoding PDM Pase of *F. moniliforme* and deduced amino acid sequence**. The stop codon is marked by an asterisk. The sequenced peptides are shown by double-headed arrows labeled with their names. Potential *N*-glycosylation sites are encircled. The splice junctions are indicated by arrowheads. Possible poly(A) addition signal is underlined.

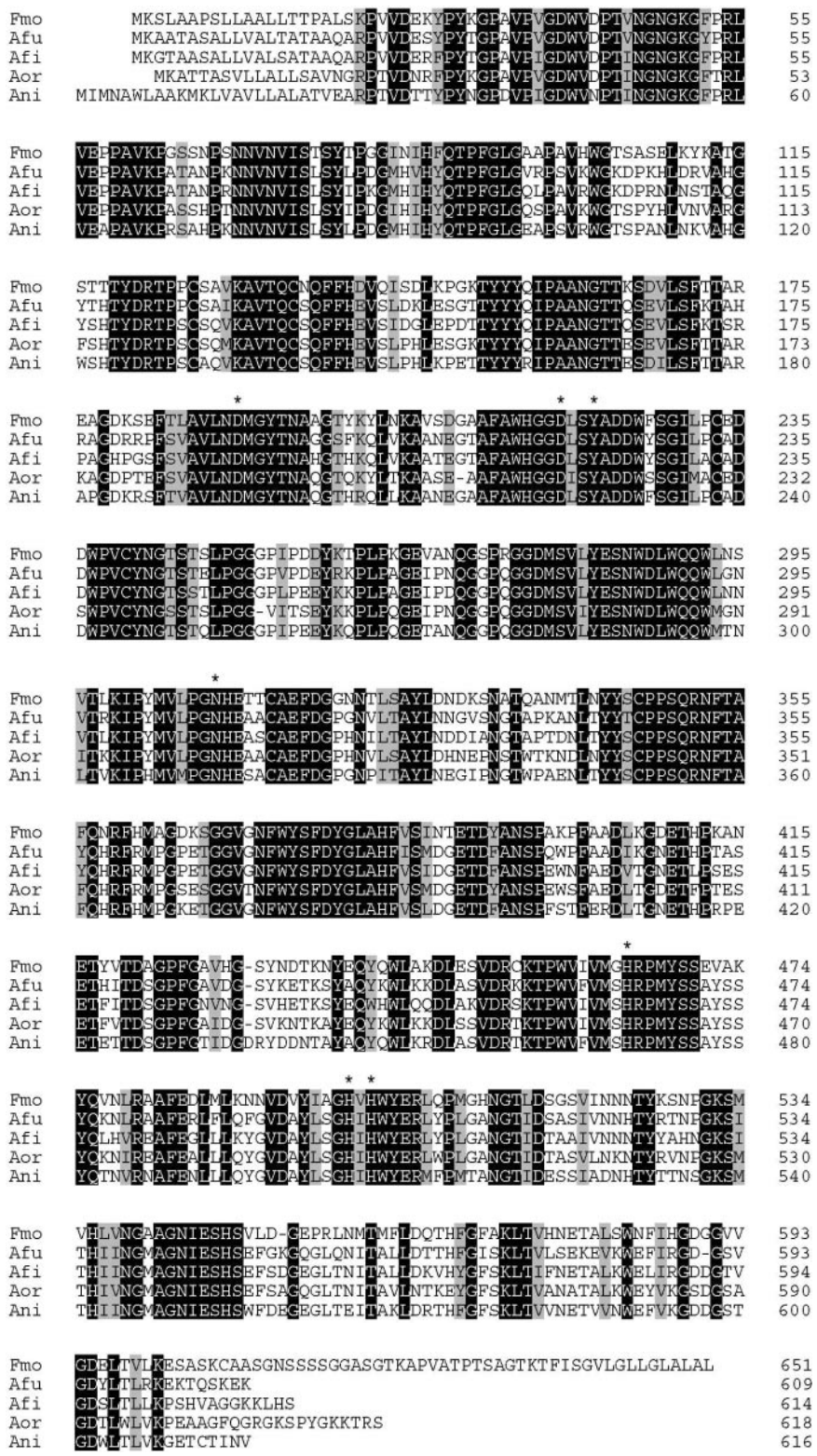

Fig. 4. **Sequence alignment of PAPs which are highly homplogous to PDM Pase.** The aligned sequences are as follows (the database name and the accession number in parentheses): Fmo, *Fusarium moniliforme* (this study); Afu, *Aspergillus fumigatus* (TrEMBL Q4WE06); Afi, *Aspergillus ficuum* (SWISS-PROT Q12546); Aor, *Aspergillus oryzae* (TrEMBL Q2TY95); and Ani, *Aspergillus nidulans* (TrEMBL Q5AR94). Fully conserved residues are highlighted in white letters against dark backgrounds. Conservatively retained residues are shadowed. Asterisks indicate those residues implicated in coordination to the binuclear metal center.

portion could be eliminated at this step. Another improvement was the use of SP-Toyopearl in place of P-cellulose, which was vulnerable to cellulase contained in the starting material Toyocelase A, a commercial product of cellulase (*6*). Damage of P-cellulose caused by cellulase often led to low flow rate, especially in a large-scale experiment. SP-Toyopearl is a synthetic polymer-based ion exchanger and has good properties in view of adsorption capacity, resolution power, flow rate, *etc*. However, gradient elution in this chromatography gave very extended activity peaks with a low yield, presumably due to denaturation by dilution. Therefore, we carried out step-wise elution and obtained a mixture of multiple forms with an acceptable yield. The last improvement was introduction of

chromatoforcusing instead of isoelectric focusing, because the former is more suitable to handle a large amount of protein. With these improvements, we could obtain the final preparation in the order of a few hundred mg. Regrettably, the preparation turned out to contain almost equal amount of another protein with a molecular mass of 58 kDa as described in "RESULTS." The new method, however, provides a convenient way to copurify the two proteins.

Based on the amino acid sequence information obtained on the purified preparation, we have successfully cloned and sequenced the full-length cDNA and the genomic DNA encoding PDM Pase. We consider that the gene encoding PDM Pase occurs probably as a single copy in the genome of *F. moniliforme* from the following reasons. Virtually only one molecular species was isolated more than once for both the cDNA and genomic DNA. Furthermore, Southern blot analysis of the *F. moniliforme* DNA with the following restriction enzymes always gave a single band of the gene with approximate lengths in kbp shown in parentheses: *Bal*I (9), *Eco*RI (20), *Eco*RV (4.5), *Mun*I (9.5), and *Stu*I (5). Therefore, as discussed earlier (*7*), multiple forms with different isoelectric points might be caused by post-translational modification, presumably glycosylation. The cDNA is regarded to correspond to the native mRNA, because all the obtained cDNAs had the same 5′-terminus and a probable TATA box (TTTAAA) was found at positions from –25 to –20 in the gene. A possible poly(A) addition signal ATAATT was found 24 b upstream of the poly(A) addition site. Although this is not standard AATAAA, such irregularity has been frequently observed in cDNAs of *F. moniliforme* (*13* and direct submission to DDBJ Data Bank, accession numbers AB071859, AB071861, AB071862, and AB092343).

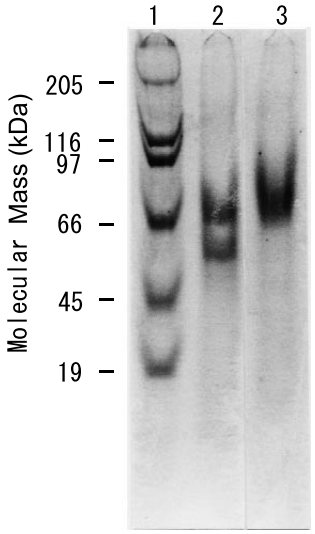

Fig. 5. **SDS-PAGE of the purified PDM Pase preparation treated with HF.** Lane 1, molecular mass standard proteins; lane 2, the sample after HF treatment; lane 3, the sample before HF treatment. Each 50 μg of the samples were loaded.

The amino acid sequence of PDM Pase deduced from the cDNA consists of 651 residues with 13 potential *N*-glycosylation sites. This corroborates the previous finding that PDM Pase is an *N*-glycosylated high mannose type of glycoprotein (*7*). Some of these sites (N161, N352, and N415) were unidentified in the peptide sequencing, suggesting that these residues were actually glycosylated. However, others (N512 and N523) were identified as such indicating that not all the potential sites are glycosylated. The deduced amino acid sequence included a number of the tryptic peptides which had been sequenced. However, not all the peptides were accounted for by the sequence, suggesting presence of other protein(s) in the purified PDM Pase preparation. Thus, we had to reinvestigate its purity. The first attempt using two-dimensional electrophoresis was ambiguous: it only gave a skew spot extending in both dimensions, suggesting presence of more than one protein or still remaining microheterogeneity, or both. The analysis was hampered by extension of the spot presumably due to different degrees of glycosylation. In fact, chemical deglycosylation of the preparation with HF and subsequent SDS-PAGE analysis revealed two clear-cut protein bands at molecular masses of 58 and 68 kDa in nearly 1:1 quantity ratio (Fig. 5). The protein with the higher molecular mass must be PDM Pase, because the mass is close to that deduced from the

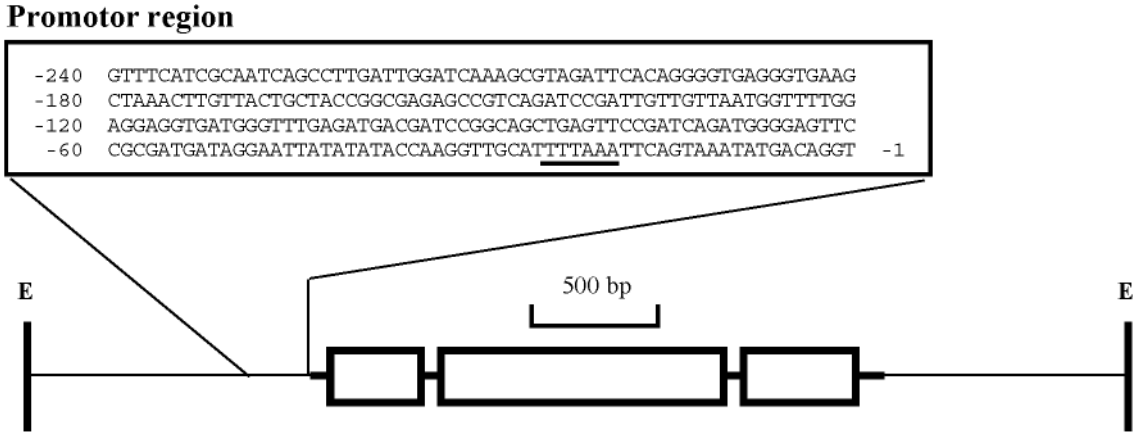

Fig. 6. **Schematic representation of PDM Pase gene and nucleotide sequence of the promoter region.** Numbering for the cDNA is applied to the promoter region, the immediate upstream position being –1. The probable TATA box is underlined. Open boxes show exons. Thick lines represent the untranslated regions of the cDNA and introns. Vertical lines labeled E indicate the *Eco*RV sites.

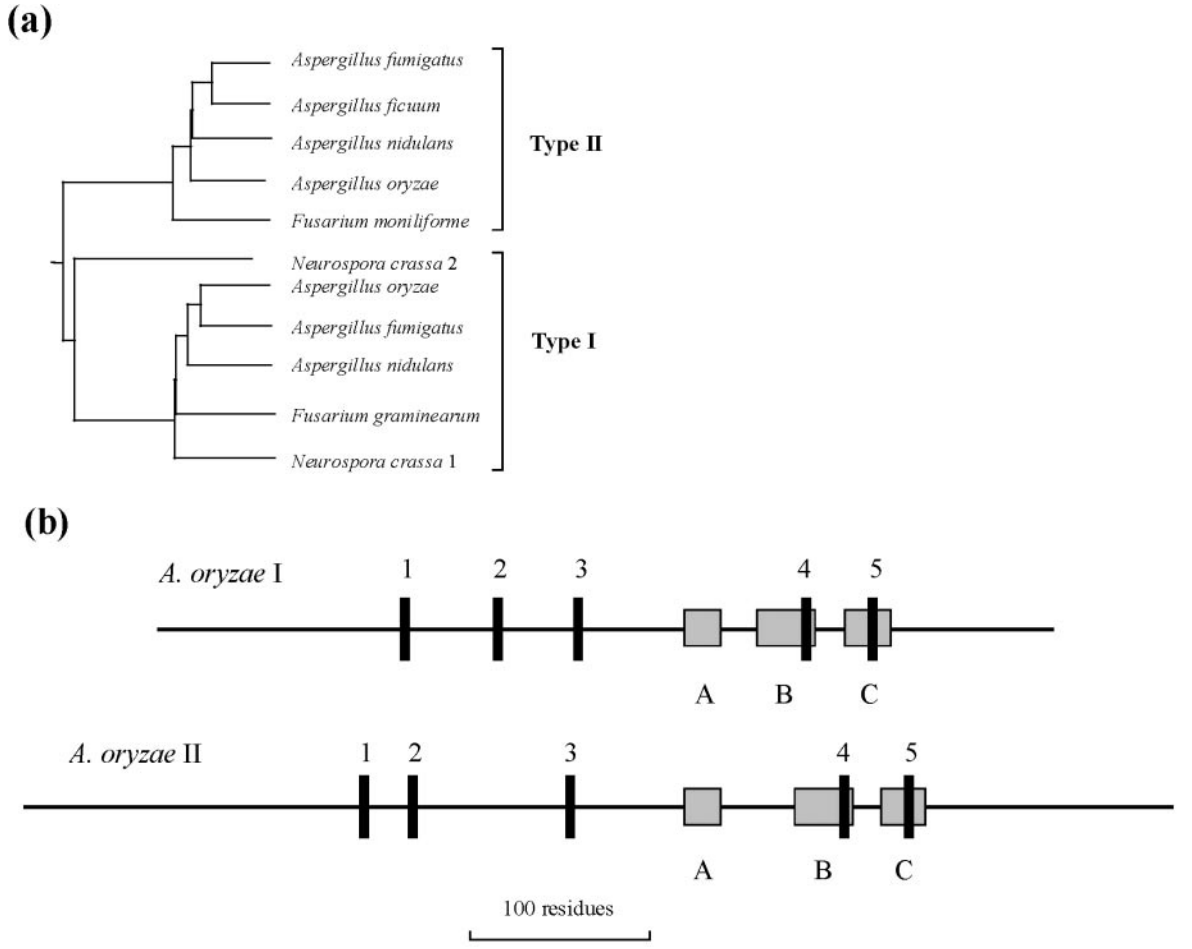

Fig. 7. **(a) Phylogenetic tree of fungal PAPs.** Type II enzymes are those cited in Fig. 4. Type I enzymes are as follows (the database name and the accession number in parentheses): *Neurospora crassa* 1 (TrEMBL Q7S852) and 2 (TrEMBL Q7S274); *Fusarium graminearum* (TrEMBL Q4IE54); *Aspergillus nidulans* (TrEMBL Q5BAS0); *Aspergillus fumigatus* (TrEMBL Q4WAC4); *Aspergillus oryzae* (TrEMBL Q2UII9). The sequence of *A. fumigatus* type I PAP had an N-terminal extension consisting of 124 residues, which broke the homology significantly. However, we judged that this was caused by an error in assignment of the initiation codon. Therefore, alignment was made after the extension was eliminated. **(b) Schematic representation of similarity between types I and II PAPs of *Aspergillus oryzae*.** Shaded boxes represent well conserved regions A, B, and C, whose consensus sequences are shown below. The two sequences are aligned so that regions A come to the same position. Vertical bars show the elements 1–5 that are implicated in coordination to the binuclear metal center by homology. Their sequences are shown with the liganding residues in bold letters.

cDNA. Our concern after the purification was that the specific activity (27.9) of the final preparation was considerably lower than that (77.4) of the most purified one reported previously (*6*). After some unsuccessful attempts, we gave up further purification and proceeded to the cDNA cloning assuming mistakenly that the low specific activity was due to partial inactivation. However, the final preparation turned out to contain almost equal amount of another protein. Had it been eliminated, the specific activity would have been doubled approaching the highest value ever reported. These results show that the contaminant protein behaved exactly in the same way as PDM Pase throughout the 500-fold purification. In other words, this protein was copurified with PDM Pase. Identity of this protein will be reported elsewhere. The reason for the copurification of the two proteins is not understood at present. Obviously, they have similar physicochemical properties as demonstrated by the failure of clear-cut separation by two-dimensional electrophoresis under the specified conditions. The copurification may simply be reflection of their very similar physicochemical properties. However, interaction between them cannot be ruled out under certain conditions such as high salt concentration, though it is unlikely that they form a tight complex judging from the elution position in

Table 3. **Properties of type II PAPs from fungi.**

	F. moniliforme	*A. ficuum*	*A. nidulans*
Physicochemical properties			
Molecular mass	100 kDa	85 kDa	100 kDa
Subunit structure	Monomer	Monomer	ND[a]
Carbohydrate	*N*-linked high Man	*N*-linked high Man	ND
Color	Faintly purple	Purple	No description
Enzymatic properties			
Optimum pH	5.3	6.0	6.1
Substrate specificity			
Phosphomonoesterase	+	+	+
Phosphodiesterase	+	+	–
Pyrophosphatase	+	+	ND
2′,3′-Cyclic nucleotidase	+	ND	ND
Nuclease	+	ND	ND
Inhibitor			
Phosphate	+	+	+
L(+)-Tartrate	–	–	–
F^-	+	–	+
Zn^{2+}	+	+	ND
Cu^{2+}	+	+	ND
EDTA	–	–	–
References	*1–7*	*18*	*19*

[a]ND, not determined.

the Sepharose 6B gel filtration. If they had formed a complex, it should have been eluted much earlier because its molecular mass would have approached 200 kDa.

The amino acid sequence has clearly shown that PDM Pase belongs to the PAP family. The seven amino acid residues implicated in coordination to the binuclear metal center, a hallmark of PAP, are all conserved (Fig. 4). This is quite unexpected to us, because we have never realized in the long course of our studies on this enzyme that it is colored purple. However, at the final step of the purification in this study, we came to notice its color for the first time. When the purified enzyme was adsorbed to a small column of phenyl-Sepharose for concentration, a dark violet band appeared at its top. The color, however, was not very deep and quickly disappeared as the elution went on. The eluate (*ca*. 3 mg protein/ml) did not appear colored in naked eyes nor did it show any absorption band in the visible region. Therefore, we concluded that PDM Pase is only faintly purple. The color is so faint that it is not recognized under usual conditions. A colorless enzyme of the PAP family is not unprecedented: Yoneyama *et al.* have reported a colorless enzyme of red kidney bean belonging to the family (*16*).

Schenk *et al.* pointed out that PAP family enzymes are widely distributed in plants, mammals, fungi, and even bacteria, and established a phylogenetic tree of these enzymes (*8*). Although they differ in size and exhibit low sequence homology between kingdoms, all the residues implicated in coordination to the binuclear metal center are conserved. Their phylogenetic tree, however, included only a few fungal PAPs. Since then, a number of fungal PAP genes have been reported. Multiple alignment of these fungal PAPs revealed that there are two types: type I with approximately 500 amino acid residues and type II including PDM Pase with more than 600 amino acid residues. The phylogenetic tree derived from the alignment is shown in Fig. 7a and schematic representation of both types of PAP from *Aspergillus oryzae* is presented in Fig. 7b. Intra-type identities generally exceed 60%, but inter-type ones are only around 20%. The only exception is *Neurospora crassa* I-2, which shows nearly 30% identities with all the other fungal PAPs. Interestingly, *N. crassa* and *F. graminearum* have only type I PAP, whereas the fungi of *Aspergillus* genus have both types. It should be recalled that the whole genome sequences are known for all these fungi. A question arises then whether the former fungi really lack type II PAP or it is merely a consequence of incomplete annotation of the genomes. As for *N. crassa*, enzymological studies on nonspecific Pases have been abundant and yet any enzyme with substrate specificity similar to that of PDM Pase has not been reported. Therefore, we do not consider that this fungus possesses type II PAP homologous to PDM Pase. On the other hand, no study has been reported on nonspecific Pases of *F. graminearum*, which belongs to the same genus as *F. moniliforme*. Therefore, discussion on PAP of *F. graminearum* is possible only at the gene level for the moment. A type I PAP has solely been registered as a hypothetical protein. However, we cannot tell at present that its genome really lacks a gene encoding type II PAP, a homolog of PDM Pase. In this regard, it should be mentioned that only a small number of Pase genes have been reported in the genome of *F. graminearum*, most of which are those encoding serine/threonine protein Pases. Probably, the *F. graminearum* genome is poorly annotated with regard to Pases. Therefore, it is possible that the gene is merely not yet identified. It is also not clear whether *F. moniliforme* lacks type I PAP. A gene encoding type I PAP, even if present, may have escaped our screening, because identity between these two types of PAPS are rather low. However, a previous study at the enzyme level has shown that PDM Pase accounts for most of the Pase activity detected in the

culture medium of *F. moniliforme* (*1*). Although this favors the hypothesis that the fungus has only the gene encoding type II PAP or PDM Pase, it is still possible that the fungus has the type I PAP gene with repressed expression. Therefore, we cannot draw any decisive conclusion at present on the absence of the type I PAP gene in *F. moniliforme*.

Studies on fungal PAPs at the protein level are not abundant. Jacobs *et al*. purified a repressible acid Pase from *N. crassa* and reported that it exhibited slight purple color (*17*). Presumably, this enzyme is one of the type I PAPs of the fungus, but nothing more is known. Therefore, most of the type I enzymes remain hypothetical proteins at present. On the other hand, three out of the five type II PAPs have been studied at the protein level. They are PDM Pase of *F. moniliforme*, extracellular pH 6.0 optimum acid Pase of *A. ficuum* (*18*), and acid Pase V of *A. nidulans* (*19*) whose properties are summarized in Table III. In spite of their very similar amino acid sequences, they differ considerably in some key properties. *A. ficuum* PAP has been reported to be purple with an absorption maximum at 580 nm ($\varepsilon_{580} = 1.3 \times 10^3$ M^{-1} cm^{-1}). However, as described above, PDM Pase is only faintly purple. If it had a molecular absorbance similar to that of *A. ficuum* PAP, we could have easily detected absorption in the visible region with our most concentrated enzyme solution. No mention was made on the color of the *A. nidulans* enzyme. With regard to enzymatic properties, we have been arguing that the most remarkable characteristic of PDM Pase is its versatile specificity toward various phosphate esters (Table 3). Although the substrate specificity has not been fully examined for the other enzymes, *A. ficuum* PAP has been reported to exhibit phosphodiesterase and pyrophosphatase activities. However, the *A. nidulans* enzyme has been shown not to possess phosphodiesterase activity. We cannot tell for the moment that these differences in the substrate specificity are caused by subtle changes in the amino acid sequences: *e.g.* PDM Pase has exceptionally long C-terminal tail. More studies will be necessary to fully understand structure-function relationship of this class of enzymes.

The nucleotide sequence data reported in this paper have been submitted to the DDBJ Data Bank under the accession numbers AB262089 (cDNA) and AB262090 (genomic DNA). The submission has been made under the organism name, *Gibberella fujikuroi*, whose anamorph is called *Fusarium moniliforme*. We have been using the latter name throughout our studies on enzymes of this fungus. We thank Ms. Y. Nariai, Department of Biochemistry, Division of Molecular Medicine, Faculty of Medicine and Ms. Y. Tanabe, Department of Biosignaling and Radioisotope Experiment, Center for Integrated Research in Science, Shimane University for their skillful technical assistance in peptide sequencing and DNA sequencing, respectively. We are also grateful to Dr. Y. Kikuchi, Department of Chemistry, Graduate School of Science, Tohoku University for guidance in the HF treatment. Cloning part of this study was made possible when one of the authors (H. Y.) was accepted as a short-term cooperation research fellow in years 1993–2002 in Division of Protein Chemistry, Institute for Protein Research, Osaka University. He is very grateful to Professors F. Sakiyama and K. Sekiguchi of the Division for acceptance in their laboratories.

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