An extracellular phosphatase was purified to homogeneity from the entomopathogenic fungus *Metarhizium anisopliae* with a 41.0% yield. The molecular mass and isoelectric point of the purified enzyme were about 82.5 kDa and 9.5 respectively. The optimum pH and temperature were about 5.5 and 75°C when using O-phospho-L-tyrosine as substrate. The protein displayed high stability in a pH range 3.0–9.5 at 30°C and was remarkably thermostable at 70°C. The purified enzyme showed high activity on O-phospho-L-tyrosine and protein tyrosine phosphatase substrate monophosphate (a specific substrate of protein tyrosine phosphatase). Although one peptide of the phosphatase shared identity with one alkaline phosphatase of *Neurospora crassa*, its substrate specificity and inhibitor sensitivity indicate that the enzyme is a protein tyrosine phosphatase.

Key words: entomopathogenic fungus; glycoprotein; *Metarhizium anisopliae*; protein tyrosine phosphatase; thermostable.

Phosphorylation and dephosphorylation of proteins play a key role in the regulation of signal transduction processes that control proliferation, differentiation, and transformation of eukaryotic cells. The phosphorylation state of a given protein is the result of coordinated action of protein phosphatase and protein kinase. According to their substrate specificities, divalent cation requirements, and inhibitor sensitivity, protein phosphatases are divided into the protein serine/threonine phosphatases and PTPases. On the basis of function, structure, and sequence, PTPases can be classified into four families: (i) the main group of tyrosine specific phosphatases, (ii) the VH1 related dual specific phosphatases (DSP), (iii) the low molecular weight PTPases (LMPTPs), and (iv) the dual specific cdc25 phosphatases. PTPase activity has been described in various organisms, including microorganisms, plants, animals, and human beings.

The *Metarhizium anisopliae*, a family of entomopathogenic fungus, has been investigated as a biological control agent for many years. Although there have been many reports characterizing the function of extracellular enzymes secreted by *M. anisopliae*, there is no published work describing PTPases secreted by *M. anisopliae*. In this report, we describe a thermostable extracellular phosphatase with a high pI isolated from *M. anisopliae* strain CQMa102. Although one peptide of the phosphatase shared identity with one alkaline phosphatase (CAC18282) of the *Neurospora crassa*, its substrate specificity and inhibitor sensitivity indicate that the enzyme is a PTPase.

Materials and Methods

*Microorganism and growth conditions.* *M. anisopliae* var. *acridum* strain CQMa102, isolated originally from *Ceracris kiangsu Tsai* by the Genetic Engineering Center of Chongqing University, was used in this study. The strain is deposited in the China General Microbiological Culture Collection Center (CGMCC) under access no. 0877. Conidia of *M. anisopliae* were obtained from cultures in 1/4-strength Sabouraud’s Dextrose agar solid medium at 27°C under constant light for 12 d. The fungus was cultured in an optimized liquid medium containing 0.4% casein (enzymatic hydrolysate, Sigma, St. Louis, Missouri) as the carbon source.
Tokyo, Japan), 0.05% MgSO₄, 0.05% KCl, 2% glucose, and 0.2% NaNO₃ in 50 mM MES (pH 6.0). A portion (1.0 ml) of the spore suspension containing 4–5 × 10⁷ spores per ml was used to inoculate 100 ml of growth media in 250 ml Erlenmeyer flasks. The culture was incubated on a rotary shaker (150 rpm) at 27 °C for 78 h.

Analysis of PTPase activity. PTPase activity was measured by a modification of the spectrophotometric method of C. Vincent et al. Briefly, an aliquot (0.1 ml) of the enzyme solution was incubated at 37 °C for 10 min in 50 mM MES buffer (pH 5.5) containing 5 mM O-phospho-l-tyrosine (Sigma, Munich, Germany). The reaction was terminated by the addition of 0.1 ml of 20% trichloroacetic acid and 0.1 ml of 10% mg/ml bovine serum albumin. The precipitated protein was removed by centrifugation at 13,000 × g for 10 min. Supernatant (0.05 ml) was used to measure the released inorganic phosphate by adding 0.1 ml distilled water and 0.05 ml mixture solution containing 3.6 M sulfuric acid, 0.5% ammonium molybdate, and 2% ascorbic acid. The sample was incubated at 37 °C for 30 min and the absorbance at 700 nm was measured. A unit (U) of enzyme activity was defined as the amount of enzyme needed to release 1.0 μmol Pi per min.

Protein determination. Protein concentration was determined using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Rockford, USA) with bovine serum albumin (BSA, Fluka, Buchs, Switzerland) as the standard.

Purification of extracellular phosphatase. Chromatographic procedures were performed on the BioLogic Duo-flow system (Bio-Rad, California, USA). Purification was monitored by SDS–PAGE and IEF–PAGE. All steps were carried out at 4 °C. Two liters of culture supernatant was filtered through four layers of gauze to remove the mycelium before the filtrate was centrifuged at 28,000 × g for 20 min. The resulting supernatant was loaded onto a ConA-Sepharose 4B column (Sigma, 1.2 × 8.4 cm) equilibrated with a buffer (50 mM MES, 1 mM MnCl₂, 1 mM MgCl₂, 1 mM CaCl₂, and 0.1 M NaCl; pH 6.0). The column was rinsed with 50 mM MES buffer (pH 6.0), and eluted with 50 mM MES buffer (pH 6.0) containing 0.5 mM methyl α-D-glucopyranoside (Sigma, Missouri, USA). The elution fractions were collected and dialysed for 36 h against 2 liters distilled water with five changes of water before the elution was centrifuged at 28,000 × g for 20 min. The supernatant was adjusted to pH 9.4 using solid Tris, and loaded onto a HighQ anion resin column (Bio-Rad, 1.5 × 11.3 cm) equilibrated with 20 mM Tris (pH 9.4). Through column portion was collected and dialysed for 5 h against 2 liters distilled water and adjusted to pH 8.0, then loaded onto a 25S cation resin column (Bio-Rad, 1.0 × 6.4 cm) equilibrated with 10 mM Tris–HCl (pH 8.0). The elution was carried out with 80 ml linear gradient from 0 to 0.15 M of NaCl containing 10 mM Tris (pH 8.0) at a flow rate of 2 ml/min. Fractions with PTPase activity were pooled.

Estimation of molecular mass and pI. The molecular mass of the purified enzyme was calculated by SDS–PAGE following Laemmli, and 12.5% gel was used. Low molecular weight markers (Pharmacia, Stockholm, Sweden) were used for calibration. Proteins were detected by silver staining.

The pI of the enzyme was determined in a 5% vertical IEF–PAGE gel using the Mini-Protean apparatus (Bio-Rad, California, USA), with a broad pH range (3–10). After IEF, the gels were stained for acid phosphatase activity using the method described by Nagy et al. Briefly, gels were incubated for 20 min at room temperature in 100 ml solution of 150 mg 1-naphthyl phosphate (Sigma, Missouri, USA) and 50 mg fast blue RR salt (Fluka, Buchs, Switzerland) in 50 mM MES buffer (pH 5.5), then transferred to a 7% acetic acid solution. Protein was subsequently detected by silver staining.

Substrate specificity of the purified enzyme and kinetic parameters. Each dispose was carried out in triplicate. The result was expressed with the mean of a triplicate assay. Protein tyrosine phosphatase substrate monophosphate (TRDIpYETDYYRK, specific substrate of PTPase), O-phospho-l-tyrosine, O-phospho-l-serine, O-phospho-l-threonine, phytic acid, ATP, ADP, AMP, and pNPP were used as substrates. All substrates were purchased from Sigma (Missouri, USA). The enzyme activity against respective substrate except for pNPP was measured as described above.

Enzyme activity against pNPP was determined by the method of Andersch and Szezyinski. Briefly, 200 μl reaction mixture containing 5 mM pNPP in 50 mM MES buffer (pH 5.5) was incubated at 37 °C for 10 min. After this period, the reaction was stopped by the addition of 50 μl 0.5 M NaOH. Aliquots were analyzed in a spectrophotometer at 405 nm. Enzyme activity was determined with reference to a calibration curve of standard 4-nitrophenol (Sigma, Missouri, USA). A unit (U) of enzyme activity was defined as the amount of enzyme needed to release 1.0 μmol 4-nitrophenol per min.

The Kₘ and Vₘₐₓ of the purified phosphatase were determined using O-phospho-l-tyrosine as a substrate at various concentrations (0.16, 0.32, 0.48, 0.64, 0.8, 0.96, and 1.44 mM), and calculated as described by Lineweaver-Burke.

Effect of pH and temperature on PTPase activity. The optimal pH for PTPase activity was determined by preparing 5 mM O-phospho-l-tyrosine as substrate in a series of buffer solutions (50 mM acetate buffer of pH 3.0–4.5, 50 mM MES buffer of pH 5.0–6.0, 50 mM HEPES buffer of pH 6.5–7.5 and 50 mM Tris buffer of pH 8.0–9.5, interval 0.5 pH) at 37 °C for 10 min. pH stability was tested by 1 h preincubation of purified
enzyme in the buffer solutions mentioned above at 30°C and 70°C, followed by dialysis against 50 mM MES buffer (pH 5.5). Thermostat stability was determined by preincubation of purified enzyme at various temperatures (50, 60, 70, 80, and 90°C) in 50 mM MES (pH 5.5) for different time (10, 30, 60, 90, 120, and 240 min). The remaining PTPase activity of each sample was determined as described above. The results were expressed as relative percentages of activity.

Influence of phosphatase inhibitors and metal cations on the PTPase activity of the purified enzyme. Each dispose was carried out in triplicate. The result was expressed as the mean of a triplicate assay. The influence of phosphatase inhibitors (NaF, Okadaic acid [Sigma], EDTA, sodium molybdate, sodium orthovanadate, N-ethylmaleimide [Fluka], sodium tungstate, sodium tartrate, β-mercaptoethanol [Sigma], dithiothreitol [Sigma]) and cations (CaCl₂, CuSO₄, FeSO₄, MgSO₄, MnSO₄, ZnSO₄, AgNO₃, BaCl₂, and CoCl₂) at various concentrations (0, 0.5, 2.0, 5.0, 10.0, and 20.0 mM) on purified PTPase activity was determined using 5 mM O-phospho-L-tyrosine as substrate in 50 mM MES buffer (pH 5.5). The purified enzyme was incubated with the respective compound for 30 min at 37°C before the substrate was added. The remaining PTPase activity was measured at optimum pH and temperature. The results were expressed as relative percentages of activity.

Chemical deglycosylation of purified enzyme. The purified PTPase was deglycosylated using anhydrous TFMS (Sigma, Tokyo, Japan) at −20°C for 20 min. The deglycosylation was assayed by SDS–PAGE followed by silver staining.

Peptide mass fingerprinting analysis. The N-terminal of the purified phosphatase could not be analyzed by Edman degradation because its N-terminal was blocked. We identified the protein using PMF. The relevant band stained by Coomassie blue was excised from the SDS–PAGE gel. After in-gel digestion with trypsin, the PMF of the purified phosphatase was analyzed by the National Center of Biomedical Analysis, Academy of Military Medical Sciences, R. P. China. PMF was performed using MALDI-TOF-MS on a REFLEX III instrument (Bruker, Bremen, Germany). Peptide product ion spectra generated by MALDI-TOF-MS were searched against the NCBI non-redundant protein database using the MASCOT search engine. The identified peptide sequence was analyzed using NCBI BLAST.

Results

Purification, molecular mass, pI, and glycosyl quantity of the extracellular phosphatase

The purification procedure for PTPase is summarized in Table 1. PTPase from M. anisopliae was purified by affinity chromatography on ConA-Sepharose and ion-exchange chromatography on HighQ and 25S, with a 41.0% yield. The purified enzyme was essentially homogeneous, showing a single band on the SDS–PAGE and IEF–PAGE gels (Fig. 1). The molecular mass of PTPase was determined to be 82.5 kDa by SDS–PAGE (Fig. 1A). Isoelectric focusing showed one band at about pH 9.5, and activity staining confirmed that it was phosphatase (Fig. 1B). After deglycosylation with TFMS, the apparent molecular mass of the enzymatic protein dropped from 82.5 kDa to about 69 kDa (Fig. 1A), indicating that the carbohydrate moiety made up about 16.4% of the total mass.

Table 1. Purification of the Extracellular PTPase from M. anisopliae Strain CQMa102

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U) (O-phospho-l-tyrosine as substrate)</th>
<th>Specific activity (U·mg⁻¹)</th>
<th>Purification fold</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture medium</td>
<td>180.9</td>
<td>8.148</td>
<td>0.045</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>ConA-Sepharose 4B</td>
<td>1.361</td>
<td>6.013</td>
<td>4.418</td>
<td>98.2</td>
<td>73.8</td>
</tr>
<tr>
<td>HighQ</td>
<td>0.445</td>
<td>3.557</td>
<td>7.993</td>
<td>177.6</td>
<td>43.7</td>
</tr>
<tr>
<td>25S</td>
<td>0.385</td>
<td>3.342</td>
<td>8.681</td>
<td>192.9</td>
<td>41.0</td>
</tr>
</tbody>
</table>

Fig. 1. SDS–PAGE and IEF–PAGE of Purified PTPase from M. anisopliae.

A. SDS–PAGE of purified PTPase with silver staining. Lane 1, purified PTPase; lane 2, molecular weight marker; lane 3, deglycosylated PTPase. B. IEF–PAGE of purified PTPase. Lane 4, silver staining; lane 5, acid phosphatase activity staining of the purified phosphatase using 1-naphthyl phosphate and fast blue RR salt.
Identification of the phosphatase by MALDI-TOF-MS

Fragment mass spectra were recorded (Fig. 2). They matched the theoretical spectra in the database by the MASCOT search engine. According to the MASCOT search results, one peptide (ALGDLLELDDTVR) was identified to be a peptide found in an alkaline phosphatase (CAC18282) of *N. crassa*. The identified peptide was analyzed using NCBI BLAST. The result indicated that the peptide was identical with several alkaline phosphatases in *N. crassa* (XP_960933, XP_326869, and EAA31697) and *Schizophyllum commune* (AA25670 and AA25679), and two hypothetical proteins in *Gibberella zeae* PH-1 (XP_387551) and *Chaetomium globosum* CBS (EAQ90516).

Substrate specificity and kinetic parameters

As indicated in Table 2, the phosphatase appeared to be rather specific for phosphotyrosine residue at pH of 5.5. There was no significant phosphatase activity against phytic acid, ADP, AMP, *O*-phospho-L-serine or *O*-phospho-L-threonine. Compared with protein tyrosine phosphatase substrate monophosphate as substrate, the phosphatase activity was only 60.3% for pNPP and 9.5% for ATP. Therefore, the phosphatase is a PTPase, not an acid phytase. The $K_m$ and $V_{max}$ values of this enzyme for *O*-phospho-L-tyrosine at pH 5.5 and 70°C were 1.273 mM and 8.72 μmol·min⁻¹.

**Effect of pH and temperature on purified enzyme activity**

The enzyme had optimum activity at about pH 5.5 (Fig. 3A). Enzymatic activity decreased remarkably below pH 5.0 and above pH 6.0. Interestingly, PTPase activity was stable after incubation of the enzyme for 1 h in a pH range 3.0–9.5 at 30°C and a pH range 4.5–8.0 at 70°C; but stability decreased markedly after incubation for 1 h at pH below 4.5 or above 8.0 at 70°C (Fig. 3B). These results indicate that the purified enzyme activity is very stable through a wide pH range.

The optimum temperature for activity was determined to be about 75°C (Fig. 4A). The thermostability of PTPase activity is shown in Fig. 4B. Remarkably, phosphatase retained full PTPase activity after heating at 70°C for 6 h, but lost 31% of it’s activity after heating at 80°C for 10 min, and all it’s activity within 10 min at 90°C. Therefore, the activity of this enzyme is very stable to heat treatment at the optimum pH. This extremely high thermostabilization is a defining characteristic of the enzyme.

**Effect of phosphatase inhibitors and metal cations on PTPase activity**

No obvious effect on PTPase activity was observed when EDTA, Mg²⁺, Co²⁺, Ca²⁺, sodium tartrate, or sodium fluoride was used at concentrations between 0.5 mM and 20 mM, or when Okadaic acid was used from...
0.5 mM to 2 mM (Table 3). But PTPase activity was significantly inhibited by orthovanadate (24.5% inhibition at 5 mM), molybdate (92.2% inhibition at 5 mM), N-ethylmaleimide (76.3% inhibition at 0.5 mM), tungstate (16.3% inhibition at 20 mM), Zn$^{2+}$ (75.2% inhibition at 5 mM), Ag$^+$ (16.6% inhibition at 2 mM), Fe$^{2+}$ (34.5% inhibition at 2 mM), and Cu$^{2+}$ (32.2% inhibition at 2 mM). In addition, PTPase activity was increased by /C12-mercaptoethanol (6.0% increase at 20 mM), Dithiothreitol (7.4% increase at 20 mM), Mn$^{2+}$ (9.2% increase at 20 mM), and Ba$^{2+}$ (42.5% increase at 20 mM) (Table 3).

**Discussion**

This is the first report, to our knowledge, describing the purification and characterization of a PTPase isolated from an entomopathogenic fungus.

About 200-fold purification of PTPase from the culture supernatant of *M. anisopliae* was obtained using ConA-Sepharose 4B, HighQ, and 25S column chromatography. The phosphatase displayed good specificity for phosphotyrosine residues. There was no significant phosphatase activity against phytic acid, ADP, AMP, O-phospho-L-serine, or O-phospho-L-threonine. Compared with protein tyrosine phosphatase substrate monophosphate as substrate, phosphatase activity was only 60.3% for pNPP and 9.5% for ATP. The results indicate that the purified phosphatase is a PTPase. But the result that one identified peptide of the phosphatase by the MALDI-TOF-MS database searching shared identity with an alkaline phosphatase found in *N. crassa* suggests that the phosphatase is an alkaline phosphatase. Moreover the identified peptide shares identity with several alkaline phosphatases in *N. crassa* and *S. commune*. It has been reported that alkaline phosphatase might dephosphorylate phosphotyrosyl proteins, but it is known that alkaline phosphatase activity is completely inhibited by EDTA and is increased by Ca$^{2+}$, Mg$^{2+}$,
The PTPase activity of the purified enzyme was completely inhibited by Fe\(^{3+}\) (20 mM) and Cu\(^{2+}\) (20 mM), which is agreement with a PTPase reported by K. Jin-Hahn et al.\(^{44}\) Altogether, all the data suggest that the phosphatase is a PTPase.

Some characteristics of the PTPase such as pI, molecular mass, kinetic parameter, thermostability, and pH stability were analyzed. The purified PTPase is stable at up to 70 °C for 240 min, and maintains 68% of its activity during 80 °C incubation for 10 min at pH 5.5. The PTPase is much more thermostable than other PTPase reported by Y. Romischki,\(^{35}\) and consistent with a PTPase purified from boar seminal vesicle glands.\(^{40}\) Moreover, the enzyme is very stable in a wide range of pHs from 3.0 to 9.5 at 30 °C and from 4.5 to 8.0 at 70 °C. Thus, extremely high stabilization is the key characteristic of the enzyme. In addition, the PTPase from *M. anisopliae* studied here exhibited a pI of 9.5, much higher than that of other PTPase.\(^{40}\) The *K_m* of the PTPase for O-phospho-L-tyrosine appears to be relatively high, but the experimentally determined *K_m* value did not deviate significantly from those previously determined.\(^{9,40}\)

In entomopathogenic fungi, this secretory PTPase might function in the utilization of phosphoproteins from dead or decaying organic matter during the saprophytic stage, but if secreted into the host, it might also play a role in pathogenesis by dephosphorylation of host phosphoproteins. Hence the purification and characterization of the PTPase in this study provides a foundation for further functional analysis of this enzyme in the organism.

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**References**


