Mycoscience 37: 65–70, 1996

Purification and characterization of secreted acid phosphatase under phosphate-deficient condition in *Pholiota nameko*

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Accepted for publication 18 December 1995

Activity of acid phosphatase secreted by mycelia of *Pholiota nameko* on cultivation for 30d in Pi-depleted medium was 88-fold higher than the corresponding activity in the Pi-supplied medium. One isozyme of the secreted acid phosphatases was purified from the culture filtrate of Pi-depleted medium by ammonium sulfate fractionation and cation exchange chromatography. The purified enzyme was homogeneous on electrophoresis. Gel filtration analysis showed that the native molecule had a molecular weight of 117,000. The molecular weight on gel electrophoresis with SDS was 52,000, indicating that the native form of the enzyme was a homodimer. The optimum pH and temperature of the enzyme were 5.5 and 45°C, respectively, and the isoelectric point of the enzyme was pH 6.9. Adsorption on Con A-Sepharose and periodic-Schiff stain suggested that the enzyme is a glycoprotein. The enzyme hydrolyzed a wide variety of phosphate esters, nucleoside phosphates, sugar phosphates, and phosphorylated amino acids. Cu^{2+}, Fe^{2+}, Hg^{2+}, iodoacetate, molybdate, tartaric acid, and SDS inhibited the enzyme activity. Fe^{3+} (1 mM), Triton X-100, methanol, and ethanol activated it. Fifteen residues of the N-terminal amino acid sequence were determined.

Key Words——acid phosphatase; N-terminal amino acid sequence; *Pholiota nameko*; phosphate-deficiency; purification.

Phosphate is an essential nutrient for all organisms. Plants can utilize only inorganic phosphate (Pi) as phosphate source. Fungal cells including yeasts, however, can also utilize organic phosphate compounds. When fungal cells have to use organic compounds as phosphate source, they must decompose the compounds to release Pi. Acid phosphatase (EC 3.1.3.2) catalyses the hydrolysis of a wide variety of phosphate esters in an acid environment to produce Pi. Under Pi-deficient condition, fungal cells can induce and secrete a considerable quantity of acid phosphatase (Caddick et al., 1986; Creanor et al., 1983; Dibenedetto, 1972; Elliott et al., 1986; Haas et al., 1991; Schweingruber et al., 1986, 1992). The secreted acid phosphatase is involved in the hydrolysis of phosphate compounds in the surroundings (Bielski, 1972) and in the transport of the free phosphates into the cells (Sommer, 1965). The mechanism of induction of acid phosphatase has been studied intensively in some yeasts (Koren et al., 1986; Schweingruber et al., 1992; Yoshida et al., 1989) and molds (Haas et al., 1992; MacRae et al., 1988) but a clear explanation of the mechanism has not been found.

In our preliminary experiments, we found that activity of acid phosphatase greatly increased in mycelia of *Pholiota nameko* (T. Ito) S. Ito et Imai under Pi-deficient condition. To our knowledge, the ratios of the activities in the mycelia and in the culture filtrate of Pi-depleted cultivation to the corresponding activities of Pi-supplied cultivation are the highest yet reported.

In the present study, to solve the mechanism of induction of acid phosphatase, we first showed the increase of this activity in the medium during Pi-depleted cultivation of *P. nameko.* Next, we tried to purify Pi-deficiency-inducible acid phosphatase from the culture filtrate of this fungus. In the process of purification, the existence of at least two isozymes was suggested and one of them was isolated and characterized.

Materials and Methods

Culture condition The strain N114 of *P. nameko* (Tohoku Shiitake Ltd.) was used in the present experiment. Mycelia were routinely subcultured every month into the Pi-supplied medium, which was composed of glucose, 20.0 g; vitamin free casamino acids (Difco), 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.27 g; KH_2PO_4, 0.2 g; $CaCl_2$, 0.1 g; $FeSO_4 \cdot 7H_2O$, 10 mg; thiamin·HCl, 10 mg; $ZnSO_4 \cdot 7H_2O$, 3 mg; $MnSO_4 \cdot 5H_2O$, 3 mg; $CuSO_4 \cdot 5H_2O$, 1 mg; $(NH_4)_6Mo_7O_{24} \cdot 7H_2O$, 1 mg; in 1 L of distilled water, pH 6.5. The Pi-depleted medium was the same as the Pi-supplied medium except for the omission of KH_2PO_4. The mycelia were inoculated into 30 ml of the Pi-supplied or Pi-depleted medium in a 200-ml Erlenmeyer flask. The cultures were grown at 25°C for 30 d in darkness.

Preparation of crude enzyme For purification of Pi-deficiency-inducible acid phosphatase, the culture fluid was filtered through a nylon mesh (#120) and the resulting solution was dialyzed against 10 mM sodium acetate

buffer, pH 5.5, and then used as a crude enzyme for further studies.

Enzyme and protein assays Acid phosphatase activity was assayed in 250 μl of reaction mixture containing 0.1 M sodium acetate buffer, pH 5.5, 0.01% Triton X-100, 10 mM β-glycerophosphate and an appropriate amount of enzyme. The mixture was incubated at 37°C for 30 min, then the reaction was stopped by adding 250 μl of 30% (w/v) trichloroacetic acid. The liberated phosphate was measured by the method of Nakamura (1950). One unit of the enzyme activity was defined as the amount of enzyme releasing 1 μmole of Pi per min under the assay conditions. Protein concentration was determined according to the method of Bradford (1976) using Bio-Rad prepared reagent (Bio-Rad) with bovine serum albumin as standard.

Gel electrophoresis SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and native-polyacrylamide gel electrophoresis (native-PAGE) were carried out according to Laemmli's method (1970) in a 10% polyacrylamide gel with or without 0.1% (w/v) SDS, respectively. Proteins were visualized by silver stain (Oakley et al., 1980). The molecular weight of protein was estimated by comparing its relative mobility on SDS-PAGE with those of standard proteins of bovine albumin (66,000), egg albumin (45,000), carbonic anhydrase (29,000), and trypsinogen (24,000).

Estimation of molecular weight by gel filtration The molecular weight of native protein was also estimated by gel filtration on a Sephadex G-100 column chromatography calibrated with β-amylase (200,000), alcohol dehydrogenase (150,000), carbonic anhydrase (29,000), and cytochrome C (12,400). The column was equilibrated and eluted at 0.1 ml/min with 10 mM sodium acetate buffer, pH 5.5, containing 0.1 M NaCl.

Detection of glycoprotein After electrophoresis, glycoproteins were stained with periodic acid-Schiff reagent (Zacharius et al., 1969). Con A-Sepharose chromatography was carried out as follows. The enzyme solution was applied to a Con A-Sepharose column (1.5 × 10 cm) equilibrated with 10 mM sodium acetate buffer, pH 5.5, containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$. After the column had been thoroughly washed with the same buffer, the adsorbed proteins were eluted with a linear gradient of α-methyl-D-mannoside from 0 to 0.5 M.

N-terminal amino acid sequence After SDS-PAGE, the protein of interest was transferred to a clear blot membrane-P (Atto) by electroblotting. Amino acid sequence was determined with an amino acid sequencer (Applied Biosystems, model 473A).

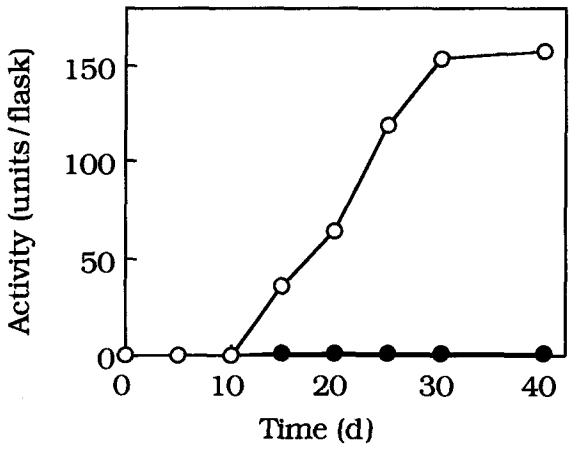

Fig. 1. Changes in activity of acid phosphatase in the culture filtrate during cultivation. ●, Pi-supplied culture; ○, Pi-depleted culture. Data were means of three replicates.

Results

Secretion of acid phosphatase under Pi-deficient condition Changes in activity of acid phosphatase in the culture filtrate during cultivation in the Pi-supplied and Pi-depleted media were examined. As shown in Fig. 1, the activity in culture filtrate was very low in both cultures for the first 10 d. Thereafter, the activity in the Pi-depleted culture increased remarkably up to 30 d, while that in the Pi-supplied culture remained at low level throughout cultivation. The activity in the Pi-depleted culture at 30 d was 88-fold higher than the corresponding activity in the Pi-supplied culture.

Purification of acid phosphatase Acid phosphatase secreted under a Pi-deficient condition was purified from the culture filtrate after 30 d of cultivation. The purification procedure is summarized in Table 1. All operations were performed at 0-4°C.

Solid ammonium sulfate was added to the crude enzyme solution to 30% saturation. The mixture was gently stirred for 1 h and the precipitate was removed by centrifugation at 10,000 × g for 30 min. The supernatant was brought to 80% saturation of ammonium sulfate. The precipitate was collected by centrifugation at 10,000 × g, dissolved in a small volume of 10 mM sodium acetate buffer, pH 5.5, and then dialyzed overnight against the same buffer.

The enzyme solution was then loaded on a CM-Toyopearl column (1.5 × 40 cm) previously equilibrated with 10 mM sodium acetate buffer, pH 5.5. The column was thoroughly washed with the same buffer to elute the

Table 1. Summary of purification of an acid phosphatase secreted from mycelia of *Pholiota nameko*.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude enzyme	13.4	3176	237	1.0	100
$(NH_4)_2SO_4$ fractionation (30–80% saturation)	5.82	909	156	0.6	29
CM-Toyopearl column	0.06	26	431	1.8	0.8

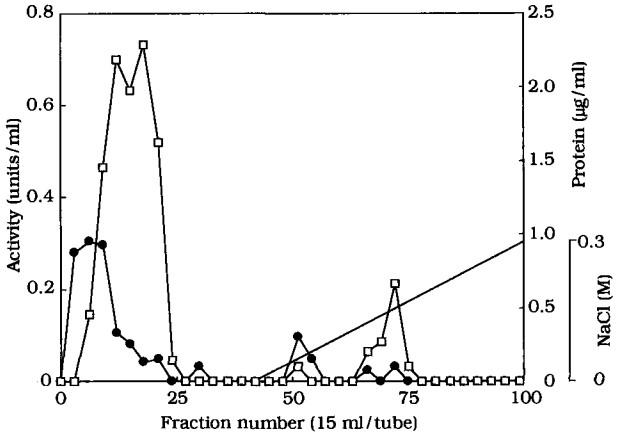

Fig. 2. CM-Toyopearl chromatography of acid phosphatase secreted from mycelia of *P. nameko*. The $(NH_4)_2SO_4$ precipitate of crude enzyme was applied to a CM-Toyopearl column (1.5×40 cm) equilibrated with 10 mM sodium acetate buffer, pH 5.5. The adsorbed proteins were eluted from the column with a linear gradient of NaCl from 0 to 0.3 M in the buffer. □, acid phosphatase activity; ●, protein; —, concentration of NaCl.

nonadsorbed proteins, while the adsorbed proteins were eluted from the column with a linear gradient of NaCl from 0 to 0.3 M. The elution profile from the CM-Toyopearl column is shown in Fig. 2. Most of the proteins charged onto the column were appeared in nonadsorbed factions. Two active fractions of acid phosphatase were obtained: one nonadsorbed (No. 6-24) and one adsorbed (No. 66-75).

Electrophoresis of acid phosphatase preparations The homogeneity of the acid phosphatase preparations was examined by native-PAGE and SDS-PAGE. The enzyme of the adsorbed fractions gave a single band on both native-PAGE and SDS-PAGE (Fig. 3). The molecular weight of the subunit protein was estimated to be 52,000 by SDS-PAGE. On the other hand, the preparation of the nonadsorbed fractions gave several protein bands both on native-PAGE and on SDS-PAGE (data not shown), showing contamination by other proteins. Thus, the purified enzyme of the former fraction was further studied as one isozyme of the secreted acid phosphatases.

Estimation of the molecular weight The molecular weight of the native enzyme was estimated to be approximately 117,000 by gel filtration (Fig. 4), suggesting that the enzyme is composed of two subunits of molecular weight of 52,000.

Substrate specificity Relative activities of acid phosphatase towards various substrates at 5 mM are shown in Table 2. The enzyme exhibited the highest reactivity towards *p*-nitrophenylphosphate, and a high level of activity towards nucleoside phosphates and sugar phosphates tested. It also hydrolyzed phosphorylated amino acids, suggesting a phosphoprotein phosphatase activity as reported for acid phosphatase from peanut (Sheikh, 1984). Although phytic acid, a major reserve of organic phosphate in seed, was a poor substrate, inositol-1-phosphate and inositol-2-phosphate were hydrolyzed to a significant extent. These results showed that the enzyme may be a low-specificity acid phosphatase hydrolyzing a variety of phosphate esters. The Km and Vmax values for β-glycerophosphate, *p*-nitrophenylphosphate, and

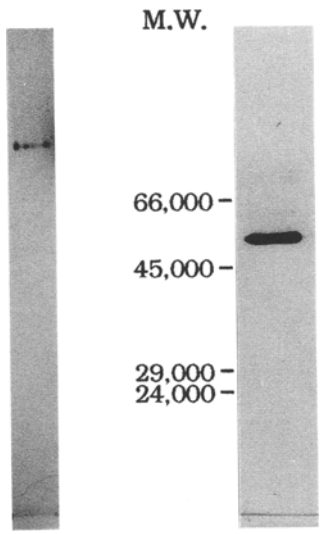

Fig. 3. Native-PAGE (A) and SDS-PAGE (B) analyses of the purified acid phosphatase. A sample of the adsorbed active fractions on CM-Toyopearl chromatography, No. 66 to 75 in Fig. 2, was submitted to electrophoresis and stained by a silver stain method. Numbers on the left side of Fig. 3B indicate molecular weights of the marker proteins.

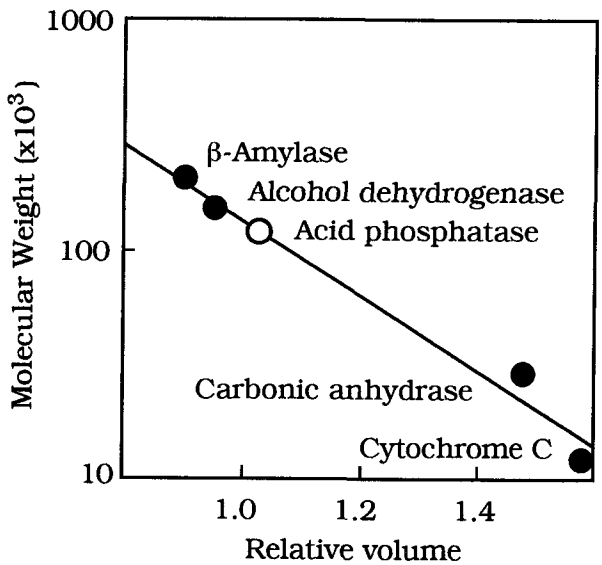

Fig. 4. Estimation of molecular weight of the purified acid phosphatase by gel filtration. The purified enzyme preparation was loaded on a Sephadex G-100 column (1.5×100 cm) and eluted with 10 mM sodium acetate buffer, pH 5.5, containing 0.1 M NaCl.

Table 2. Substrate specificity of a secreted acid phosphatase.

Substrate (5 mM)	Relative activity (%)
β-Glycerophosphate	100
p-Nitrophenylphosphate	128
2'-AMP	120
ADP	88
ATP	93
Fructose-6-phosphate	106
Ribose-5-phosphate	116
Phytic acid	21
o-Phosphoserine	102
o-Phosphothreonine	83
α-Naphtylphosphate	116
Inositol-2-phosphate	35
Inositol-1-phosphate	57

ATP were determined from Lineweaver-Burk plots under the assay conditions. They were found to be approximately 2.06, 0.74, and 0.13 mM, and 0.19, 0.30, and 0.12 μmol/min, respectively.

Table 3. Effect of various chemicals on the activity of a secreted acid phosphatase.

Chemical[a)]	1 mM	10 mM
None	100	100
LiCl	100	117
NaCl	116	109
KCl	94	95
NH_4Cl	83	103
$ZnCl_2$	108	104
$CaCl_2$	109	111
$MgCl_2$	87	117
$MnCl_2$	96	109
$CuSO_4$	84	21
$HgCl_2$	9	8
$FeSO_4$	67	51
$FeCl_3$	157	84
EDTA	100	86
Ammonium molybdate	8	4
Iodoacetic acid	90	—[b)]
DTT	103	—
NEM	101	—
PCMB	107	—
NaF	112	86
Tartaric acid	95	40
Triton X-100 (0.01%)	134	
SDS (0.1%)	8	
Ethanol (10%)	195	
Methanol (10%)	130	

[a)] Abbreviations: DTT, dithiothreitol; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuric benzoate.
[b)] —, not determined.

Effect of various chemicals on enzyme activity The effect of various chemicals on the enzyme activity were examined. The results are shown in Table 3. Monovalent cations did not affect the activity. Among the metal ions tested, Cu^{2+}, Fe^{2+}, and Hg^{2+} inhibited the activity. Although Fe^{2+} seemed to be a potent inhibitor, Fe^{3+} stimulated the activity at low concentration (1 mM). Thiol blocking agents, iodoacetate, NEM, and PCMB, and a protective agent for SH groups, DTT, did not change the enzyme activity. NaF, as a typical acid phosphatase inhibitor, had no effect on the activity, while Mo ion inhibited the activity. Tartaric acid, an inhibitor of acid phosphatase from animal, inhibited the enzyme activity at high concentration (10 mM). SDS, an ionic detergent, inhibited the activity and Triton X-100, a nonionic detergent, increased it. Methanol and ethanol increased the enzyme activity as phosphate acceptors.

Other properties Table 4 summarizes the properties of the purified acid phosphatase preparation. The optimum pH of the enzyme was 5.5. The enzyme was stable at pHs between 3 and 7 when the activity was measured after incubation at 0°C for 24 h at various pHs. The optimum temperature was estimated to be 45°C. Pretreatment of the enzyme at various temperatures for 15 min revealed that the enzyme was relatively stable below 40°C, but rapidly lost the activity above 45°C. By isoelectric focusing using Ampholine (Pharmacia, pH 3.5–10) in a glycerol gradient, the isoelectric point of the enzyme was found to be pH 6.9. Since the enzyme was adsorbed on a column of Con A-Sepharose and also stained with periodic-Schiff reagent (data not shown), the enzyme seems to be a glycoprotein.

Amino acid sequence The N-terminal amino acid sequence of the purified enzyme was examined and fifteen amino acid residues were determined. The sequence was K-R-P-I-V-P-G-A-V-F-D-R-F-I-T. About 60% of the residues were hydrophobic amino acids. A computer search revealed no significant homology with protein sequences entered in the Swiss-Prot data base.

Discussion

The present study shows that mycelia of *P. nameko* secrete a large quantity of acid phosphatase in Pi-deficient condition. Acid phosphatase activity in culture filtrate was 88-fold higher in the Pi-depleted culture than in the Pi-supplied culture after 30 d. In the case of the Pi-depleted culture, up to 83% of the total activity, namely,

Table 4. Properties of a secreted acid phosphatase.

Properties	
Optimum pH	5.5
Optimum temperature	45°C
pH stability	3–7
Temperature stability	10–40°C
Isoelectric point	6.9
Carbohydrate	+

the sum of the activities in mycelia and culture filtrate, was found in the culture filtrate. Plants and fungi were also shown to increase acid phosphatase activity on Pi starvation (Creanor et al., 1983; Elliott et al., 1986; Field and Schekman, 1980; Goldstein et al., 1988; Lefebvre et al., 1990; Mitchison and Creanor, 1969; Ninomiya et al., 1977; Tadano and Sakai, 1991; Toh-e and Ishikawa, 1971). However, the amount of acid phosphatase secreted by plants under Pi-deficient condition was one fifteenth to one fiftieth of that secreted by mycelia of *P. nameko* (Goldstein et al., 1988; Lefebvre et al., 1990; Ninomiya et al., 1977; Tadano and Sakai, 1991). Yeasts increase the activity of acid phosphatase on starvation of Pi at higher levels than plants. The induced acid phosphatases were found to be mainly associated with the cell wall and only a little was released into the medium (Creanor et al., 1983; Elliott et al., 1986; Field and Schekman, 1980; Mitchison and Creanor, 1969). Due to the large production of the induced and secreted acid phosphatases, the mycelia of *P. nameko* provide an excellent system for studying the mechanism of induction and secretion of acid phosphatase under Pi-deficient condition.

On CM-Toyopearl column chromatography after ammonium sulfate fractionation of crude enzyme, two active peaks were detected, in the nonadsorbed and adsorbed fractions, suggesting that mycelia of *P. nameko* secrete at least two acid phosphatase isozymes. We purified the latter active fraction to homogeneity.

Comparison of protein profiles from Pi-depleted and Pi-supplied media by SDS-PAGE revealed that the band with molecular weight of 52,000 corresponding to the acid phosphatase was not stained in the culture filtrate of the Pi-supplied culture and found only in that of the Pi-depleted culture (data not shown). It is concluded that the enzyme is an acid phosphatase of a Pi-deficiency-inducible type.

The purified acid phosphatase showed a broad specificity towards various substrates. It also had the rare property of hydrolyzing inositol monophosphates to a significant extent (Kaufman and Kleinberg, 1975; Fujimoto et al., 1993; Yamagata et al., 1980). While dephosphorylation of inositol monophosphate by acid phosphatase has been reported in aleurone particles of rice grain (Yamagata et al., 1980) and bovine brain (Fujimoto et al., 1993), the rates of hydrolysis by the enzymes were lower than that from *P. nameko*. The acid phosphatase secreted from *P. nameko* also had low activity to phytic acid. We are not sure of the physiological significance of the inositol phosphatase activity, since inositol phosphates are rare compounds in natural woods. However, inositol phosphates are major organic phosphates in wheat bran and rice bran, which are components of artificial sawdust medium. Therefore, the acid phosphatase secreted from *P. nameko* under Pi-deficient condition can probably hydrolyze inositol phosphates, which are abundant in wheat or rice brans, and the mycelia take in the liberated Pi.

It was also suggested that the purified acid phosphatase is not a metalloprotein and that a sulfhydryl group does not play any role in the catalytic site of the enzyme or in supporting the active conformation. These properties seem to be typical features of plant and microbial acid phosphatases (Haas et al., 1991; Ullah and Cummins, 1987, 1988; Yoshida et al., 1989). Activation by Triton X-100, a nonionic detergent having a strong affinity for hydrophobic side chains, has also been observed in acid phosphatase from *Barley coleoptiles* (Stefania et al., 1992) and *Vigna sinensis* (Biswas and Cundiff, 1991). The interaction of this detergent with hydrophobic domains of the enzyme may enhance its activity. Phosphate acceptors like ethanol and methanol also activated the enzyme. By catching Pi liberated by the enzyme, the acceptors probably prevent the Pi from inhibiting the enzyme activity. Although the N-terminus of acid phosphatase is blocked in many organisms (Erion et al., 1991; Haas et al., 1991; Lee et al., 1991; Tanaka et al., 1990), that of the enzyme from *P. nameko* was not blocked.

Acknowledgements——We are grateful to Mr. Toshiyuki Sawai, Mr. Takahiro Watanabe and Mr. Yuji Tasaki for technical support.

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