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Short Communication

Acid phosphatase isozymes secreted under phosphate-deficient conditions in *Pholiota nameko*

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We previously reported the purification of an acid phosphatase (APase52) secreted from the mycelia of *Pholiota nameko* under phosphate-deficient conditions. In the present study, two other isozymes (APase47 and APase48) were found and their structures were compared with that of APase52. Thirteen amino acid residues at the *N*-terminus of APase47 were completely identical with those of APase48 and had partial homology with those of APase52. The deglycosylation of proteins indicated that three APase isozymes differ in the *N*-linked oligosaccharide content. The protease-generated peptide maps of the APases differed from one another in the band pattern. These results suggest that the APases are the products of different genes.

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

Phosphorus is an essential element for all organisms, which can absorb phosphorus in the form of an orthophosphate anion (Pi; $H_2PO_4^-$ or HPO_4^{2-}). Acid phosphatase (APase; EC 3.1.3.2) is a key enzyme in the regulation of Pi metabolism in cells. Pi deficiency causes a significant elevation of intracellular and extracellular APase activity in various plants (Elliott et al.,1986; Goldstein,1988; Lefebvre et al.,1990) and fungi (Tadano and Sakai,1991). We previously reported that activity of APase secreted by the mycelia of *Pholiota nameko* (T. Ito) S. Ito et Imai in Imai cultured under Pi-deficient conditions is 88-fold higher than the corresponding activity under Pi-sufficient conditions (Joh et al., 1996). Under Pi-deficiency, the mycelia induced and secreted several APase isozymes. We previously purified one of these, which is composed of subunits with molecular weight of 52,000 (APase52), from the culture filtrate of the Pi-deficient culture and described its physical and kinetic properties. In this study, we describe two other APase isozymes and compare their structural properties with those of APase52.

Strain N114 of *P. nameko* (Tohoku Shiitake) was used in the study. The mycelia were inoculated into 30 ml of the Pi-depleted medium described previously (Joh et al., 1996) in a 200-ml Erlenmeyer flask and cultivated at 25°C for 30 d in darkness.

The culture fluid was filtered through a nylon mesh (# 120). The filtrate was fractionated with ammonium sulfate (50–100%), and the enzyme was chromatographed on CM-Toyopearl (Tosoh, 2.8 × 16 cm) as described previously (Joh et al., 1996). Two activity peaks were observed (see Fig. 2 in Joh et al., 1996), of which the adsorbed fraction contained APase52. Here, the non-adsorbed fraction was further purified by Dye-Matrex Red A (Amicon, 2.8 × 16 cm) equilibrated with 10 mM acetate buffer (pH 5.5) after dialyzation against the same buffer. The enzyme was eluted with a linear gradient of NaCl from 0 to 1.0 M. The active fraction was finally purified by isoelectric focusing according to the method of Igaue and Watanabe (1976).

The final enzyme preparation was examined on 10% SDS-polyacrylamide gel and gave two bands of proteins at positions corresponding to molecular weights of 47,000 and 48,000 (Fig. 1A). When the gel was activity-stained after SDS-PAGE by the method of Lacks and Springhorn (1980), two bands were observed at positions corresponding to the two bands of protein (Fig. 1A). In native-PAGE analysis of the preparation, two bands of APase activity were also detected at positions corresponding to the two protein bands (Fig. 1B). These results indicate that the final enzyme preparation involve two APase isozymes and that the mycelia of *P. nameko* secrete at least three APase isozymes, including APase52, under Pi-deficient conditions. When the final enzyme preparation was analyzed by gel filtration, only one peak with APase activity was detected at the position corresponding to a molecular weight of 50,000 (data not shown). This result and the SDS-PAGE analysis described above together suggest that both APases are monomers. The APases with the estimated molecular weights of 47,000 and 48,000 were designated as APase47 and APase48, respectively.

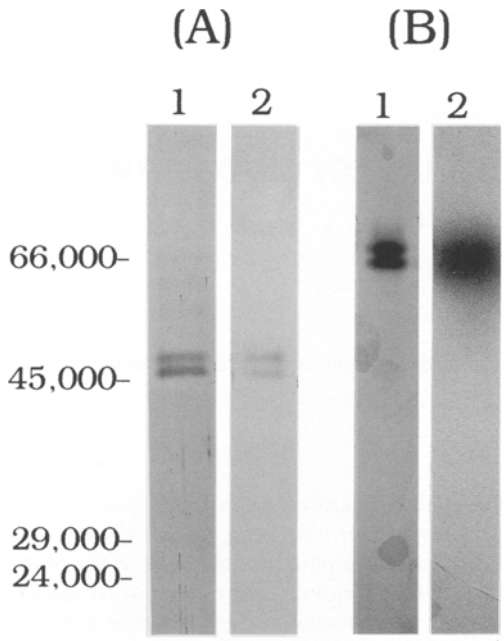

Fig. 1. SDS-PAGE (A) and native-PAGE (B) analyses of APase isozymes.
The active fraction on isoelectric focusing was submitted to electrophoresis. Lanes: 1, silver staining; 2, activity staining. Numbers on the left side of (A) indicate molecular weights of the marker proteins.

Although APase47 and APase48 could not be separated by column chromatography in various trials, they could be separated by SDS-PAGE. Therefore, the following structural studies were carried out after the electrophoresis.

APase47 and APase48 were transferred from SDS-PAGE gel to a clear blot membrane-P (Atto) by electroblotting, and the *N*-terminal amino acid sequences were determined by use of an automatic microsequencer (Applied Biosystems, model 473A). Sequence homology search was performed using GENETYX (Software Development). The *N*-terminal sequence of 13 amino acids of APase47 was completely identical with that of APase48 (Fig. 2). The sequence had partially homology with that of APase52 (Joh et al., 1996). A computer search revealed that the sequence of APase47 and APase48 showed homology with those of Pi-repressible APase precursor from *Penicillium chrysogenum* Thom (Haas et al., 1992) and APase precursor from *Aspergillus ficuum* (Reichardt) Hennings (Ehrlich et al., 1994). The former and the latter had respectively 58.3 and 50.0% homology with the sequence of APase47 and APase48 over a 12-amino acid overlap.

The structual relationship among APase47, APase48 and APase52 was investigated by peptide mapping. APase52 was purified by the method of Joh et al. (1996) and used in the experiments. The APases were excised from SDS-PAGE gel and cleaved with Staph protease (Worthington biochemical) according to the method of Cleveland (1977). The degradation products were analyzed on a 15% (w/v) SDS-polyacrylamide gel. The cleavage pattern of APase52 differed from those of

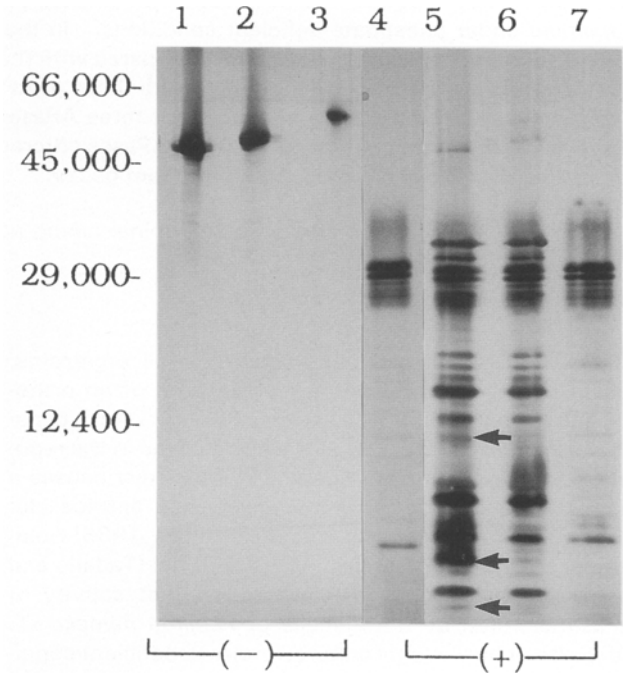

Fig. 3. Peptide map of APases.
APases were treated with (+) or without (−) Staph protease. Gel was stained with silver staining method. Lanes: 1 and 5, APase47; 2 and 6, APase48; 3 and 7, APase52; 4, Staph protease. Arrows indicate bands detected only in APase47. Numbers on the left of the figure indicate molecular weights of the marker proteins.

APase47	1	IVPG--VVFDRYVSI	13
APase48	1	IVPG--VVFDRYVSI	13
APase52	1	KRPIVPG-AV-FDRF--IT	15
PCPhoA	48	VKGLA--FDRFVNI	59
AFPhoA	48	VKGLT--FNRFVNI	59

Fig. 2. Comparison of amino acid sequences of APases.
APases shown in alignment with *Pholiota nameko* APase isozymes are *Penicillium chrysogenum* PhoA (PCPhoA) and *Aspergillus ficuum* PhoA (AFPhoA). Gaps (represented by dashes; −) were added where necessary to maximize alignment of similar residues. Shaded and boxed regions represent residues identical with APase47, and with APase52 but not with APase47, respectively.

APase47 and APase48 (Fig. 3). Several differences were also found between the peptide maps of APase47 and APase48. Three peptide bands at positions corresponding to molecular weights of 12,000, 6,500 and 5,200 were detected in APase48 but not in APase47.

Glycoprotein staining by the periodic acid Schiff reaction method (Zacharius et al., 1969) showed that the three APases are glycoproteins (data not shown). Therefore, the APases were deglycosylated enzymatically with glycopeptidase F (Takara) by modification of the procedure of Plummer et al. (1984) and Tarentino et al. (1985), and the products were analyzed by SDS-PAGE. The final enzyme preparation, the mixture of APase47 and APase48, was used in this experiment. APase (50 μg) was incubated with 2 milliunits of glycopeptidase F overnight at 37°C in 10 mM acetate buffer (pH 5.5). As shown in Fig. 4, APase47, APase48 and APase52 gave only a single band, in each case at the corresponding to a molecular weight of 42,000. Based on the molecular weights of APases after and without deglycosylation, we deduced that APase47, APase48, and APase52 differ in the *N*-linked oligosaccharide content.

The occurrence of multiple isozymes is considered to be due to either the epigenetic modification of enzyme molecules or the presence of the corresponding multiple genes. The former has been reported in alkaline phosphatase of the strain K-12 of *Escherichia coli* (Migula) Castellani et Chalmers (Garen and Garen., 1963; Nakata et al., 1977, 1978). The isozymes with the different electrical mobility are coded by a single structural gene and occur by the removal of an amino-terminal arginine residue. On the other hand, several APase isozymes of plants and fungi have been shown to be coded respectively by the different genes (Bostian et al., 1980; Loppes et al., 1978; Shimada et al., 1977; Toh-e et al., 1976). The APase isozymes of *P. nameko* are assumed to be of the latter type as judged by their structural properties. That is, because APase52 was distinct in its *N*-terminal amino acid sequence and peptide map from APase47 and APase48, and APase48 differed in its peptide map from APase47, it seems reasonable to conclude that they have distinct amino acid sequences and correspond to individual structural genes.

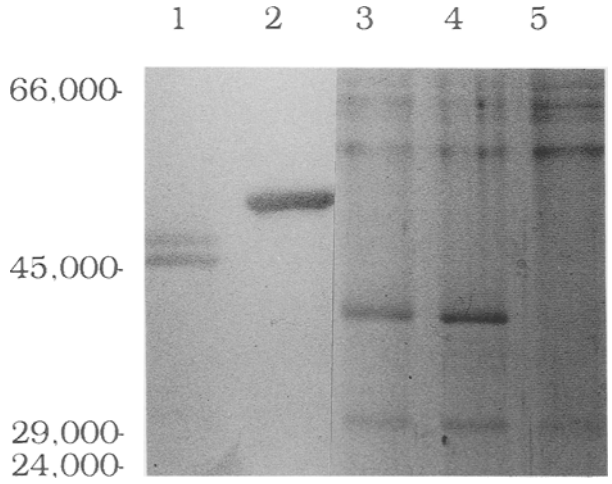

Fig. 4. SDS-PAGE of APases after or without deglycosylation by glycopeptidase F.
Lanes: 1, APase47 and APase48; 2, APase52; 3, deglycosylated APase47 and APase48; 4, deglycosylated APase52; 5, glycopeptidase F. In lanes 3 and 4, the bands corresponding to molecular weights of 55,000–60,000 arised from contaminating proteins. Numbers on the left of the figure indicate molecular weights of the marker proteins.

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