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Note

Isolation and Properties of Glucose-1-phosphatase from Mycelia of *Pholiota nameko*

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An acid phosphatase with a very high substrate specificity for glucose-1-phosphate was isolated for the first time from mycelia of *Pholiota nameko*. The molecular weight of the enzyme was estimated to be 31,000 on gel filtration and 35,000 on SDS-PAGE. The activity was inhibited by Cu^{2+}, Hg^{2+}, molybdate, and tartaric acid. The sequence of *N*-terminal 20 amino acid residues was analyzed.

Key words: acid phosphatase; glucose-1-phosphatase; mushroom; *Pholiota nameko*

Acid phosphatase (EC 3.1.3.2) is a key enzyme in the regulation of P_i metabolism. Normally, there are several isozymes of acid phosphatase, which differ in the subcellular location and the kinetic and molecular properties, in organisms. Acid phosphatases are classified as extracellular or intracellular. Most extracellular acid phosphatases are relatively non-specific enzymes, which are involved in the acquisition of P_i from the environment.[1-3] Intracellular acid phosphatases are undoubtedly important in the production, transport, and recycling of P_i.[2] Although many of them have only a low substrate selectivity in the same manner as extracellular enzymes, some enzymes have a clear substrate specificity.[4-10] These acid phosphatases with a high specificity are presumed to have more specialized metabolic functions than non-specific enzymes. However, their precise physiological roles remain unclear. In this study, we purified an acid phosphatase with a high specificity for Glc-1-phosphate from mycelia of *Pholiota nameko* and discussed its putative function.

P. nameko N114 from Tohoku Shiitake Ltd. (Sendai) was used in this study. The mycelia were inoculated into the liquid medium described previously[11] and cultivated at 25°C in darkness for 30 d. Acid phosphatase activity was assayed in 0.5 ml of a reaction mixture containing 0.1 M acetate buffer (pH 5.5), 15 mM *p*-nitrophenylphosphate, and an enzyme. The mixture was incubated at 37°C for 10 min, then the reaction was stopped by adding 1 ml of 1 M Na_2CO_3. The amount of *p*-nitrophenol liberated was measured by the absorbance at 400 nm. One unit of the enzyme activity was defined as the amount of enzyme liberating 1 μmole of P_i per min under these assay conditions. Protein was measured by the method of Bradford[12] using Bio-Rad prepared reagent (Bio-Rad) with bovine serum albumin as a standard.

All purification steps were done at 4°C. The mycelia were collected on a nylon mesh (#120), washed with 10 mM acetate buffer (pH 5.5), and then homogenized in the buffer using an Osterizer blender (Oster) at 16,800 rpm for 2 min. The homogenate was centrifuged at 10,000 × *g* for 30 min. Solid ammonium sulfate was added to the supernatant to 70% saturation. The mixture was gently stirred for 1 h and centrifuged at 10,000 × *g* for 30 min. The supernatant was brought to 90% saturation of ammonium sulfate. The precipitate was collected by centrifugation, dissolved in a small volume of 10 mM acetate buffer (pH 5.5), and then dialyzed against the buffer. The enzyme solution was put on a DEAE-Toyopearl column (1.5 × 11 cm) previously equilibrated with 10 mM acetate buffer (pH 5.5). The column was thoroughly washed with the same buffer to elute the non-adsorbed proteins, while the adsorbed proteins were eluted from the column with a linear gradient of NaCl from 0 to 0.3 M NaCl. The elution profile showed four active peaks (P1 ~ P4 in Fig. 1), suggesting that at least four acid phosphatase isozymes exist in mycelia of *P. nameko*. When each fraction was analyzed by electrophoresis by the method of Laemmli,[13] only fractions from No. 57 to 60 (P2) gave a single band on both of native-PAGE and SDS-PAGE (Fig. 2). From 800 g of the mycelia, 20 μg of the purified enzyme was obtained with a yield of 0.5%. The specific activity was

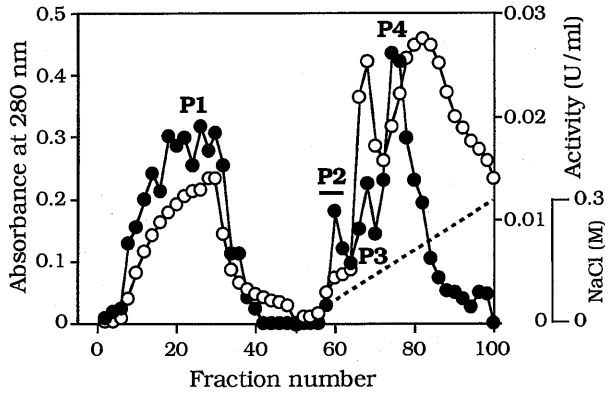

Fig. 1. DEAE-Toyopearl Chromatography of Acid Phosphatase from Mycelia of *P. nameko*.

The 70–90% $(NH_4)_2SO_4$ precipitate of crude enzyme was put on a DEAE-Toyopearl column. ●, acid phosphatase activity; ○, protein; ---, concentration of NaCl. The fractions collected as the purified enzyme preparation were indicated by solid bar.

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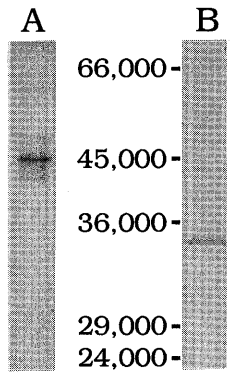

Fig. 2. Native-PAGE (A) and SDS-PAGE (B) Analyses of AP35.
The P2 fraction was electrophoresed and silver-stained by the method of Oakley *et al.*[25] Numbers on the left of Fig. 2B indicate molecular weights of the marker proteins.

8.5 U/mg. The low yield is considered to be due to separation of the isozymes on DEAE-Toyopearl chromatography, and to obtain enough enzyme to examine the properties we had to purify it several times and pool it. The subunit molecular weight of the enzyme was estimated to be 35,000 by SDS-PAGE. By gel filtration on Sephacryl S-200 HR column (2.6 × 120 cm) equilibrated with 10 mM acetate buffer (pH 5.5) containing 1.0 M NaCl, the native molecular weight of the purified enzyme was estimated to be 31,000 (data not shown). These results suggest that the enzyme is present in a monomeric form. The purified acid phosphatase was named AP35 for the molecular weight on SDS-PAGE.

The effects of pH and temperature on the activity of AP35 were examined with *p*-nitrophenylphosphate as substrate. The optimum pH of AP35 was 5.9. The enzyme was stable at pHs between 3.0 and 7.0 but rapidly lost the activity above pH 8.0. The optimum temperature of AP35 was 40°C.

The effects of various compounds on the activity of AP35 were examined. The results are shown in Table 1. Ca^{2+} stimulated the activity, while Hg^{2+} and Cu^{2+} inhibited it. Chelating reagents and thiol blocking reagents such as EDTA and *N*-ethylmaleimide had no effect on the activity, suggesting that AP35 is not a metalloprotein and that a sulfhydryl group does not play any role in the catalytic reaction of the enzyme or in supporting the active conformation. These properties seem to be typical features of plant and microbial acid phosphatases.[14-17] Among typical inhibitors of acid phosphatase, molybdate greatly inhibited the activity but NaF caused no inhibition. The activity of AP35 was also inhibited by tartaric acid, an inhibitor of animal acid phosphatase.

The activity of AP35 against various phosphorylated compounds was assayed and the K_m and V_{max} for each compound were calculated by a nonlinear regression method. The enzyme was incubated in 150 μl of 0.1 M acetate buffer (pH 5.5) containing various compounds at 37°C for 10 min, then the reaction was stopped by adding 150 μl of 30% (w/v) trichloroacetic acid. The liberated phosphate was measured by the method of

Table 1. Effects of Various Compounds on the Activity of AP35

Compounds (10 mM)	Relative activity (%)
None	100
$CaCl_2$	123
$MgCl_2$	99
$ZnCl_2$	83
$CuSO_4$	45
$HgCl_2$	1
EDTA	104
N-Ethylmaleimide	95
$(NH_4)_6Mo_7O_{24}$	2
NaF	114
Tartaric acid	64

Table 2. Substrate Specificity of AP35

Substrate	K_m* (mM)	V_{max}* (mol/min/mg)	Specificity constant (V_{max}/K_m)
p-nitrophenylphosphate	2.2±0.4	0.03±0.00	0.014
Glc-1-phosphate	4.8±1.5	2.36±0.23	0.491
Glc-6-phosphate	N.D.	N.D.	N.D.
Gal-1-phosphate	85.4±3.4	2.09±0.07	0.025
Gal-6-phosphate	N.D.	N.D.	N.D.
Fru-1-phosphate	12.2±0.8	0.13±0.00	0.010
Fru-6-phosphate	14.3±2.0	0.10±0.01	0.007
Rib-5-phosphate	7.9±1.2	0.03±0.00	0.004
AMP	N.D.	N.D.	N.D.
ADP	13.4±2.3	0.21±0.02	0.015
ATP	56.7±8.6	0.41±0.15	0.007
Phosphoenolpyruvate	12.3±3.1	0.04±0.01	0.003
β-Glycerophosphate	N.D.	N.D.	N.D.
α-Naphtylphosphate	N.D.	N.D.	N.D.

N.D.; The kinetic parameters could not be measured because of low activity.
*; Data are indicated along with standard error.

Nakamura.[18] Table 2 lists the K_m and V_{max} along with the specificity constants (V_{max}/K_m) for the compounds used as substrates. The K_m was the lowest for the synthetic substrate, *p*-nitrophenylphosphate, and secondly low for Glc-1-phosphate. The highest V_{max} was found with Glc-1-phosphate. These kinetic constants of AP35 led to the highest specificity constant for Glc-1-phosphate, at least 20-fold greater than the value obtained for any other compounds. The enzyme also have little activity against Glc-6-phosphate, Gal-6-phosphate, AMP, β-glycerophosphate, or α-naphthylphosphate. These results indicate that the substrate specificity of AP35 is clearly different from many non-specific acid phosphatases and has a very high substrate specificity for Glc-1-phosphate. It can therefore be presumed that the enzyme plays some role in P_i or carbohydrate metabolism as Glc-1-phosphatase. To our knowledge, the reports of Glc-1-phosphatase have been made only for *Escherichia coli*.[19-21] Since the K_m of the enzyme for Glc-1-phosphate has been reported to be 0.5 mM, one thirtieth lower than the values for tested any other compounds, the enzyme from *E. coli* has much higher affinity for Glc-1-phosphate than that from *P. nameko*. Glc-1-phosphatase of *E. coli* is present in the periplasmic space and the physiological function is definitely not the

use of phosphate from Glc-1-phosphate but that of the glucose moiety. Although the localization of AP35 in *P. nameko* is still uncertain, AP35 may be involved in the use of exogenous Glc-1-phosphate as a carbon source rather than as a phosphate source in the same manner as Glc-1-phosphatase in *E. coli*, because it was found in the mycelia grown in P_i-sufficient medium. Alternatively, Glc-1-phosphate is an important substance as a metabolite of the main carbohydrate, trehalose, and the storage polysaccharide, glycogen, in mushrooms.[22] Therefore, AP35 may play some role in the carbohydrate metabolism in *P. nameko*. Gal-1-phosphatase from rat brain also hydrolyzes Glc-1-phosphate at a considerable rate.[23] However, the enzyme is widely different from AP35 of *P. nameko* in optimum pH, need of Mg^{2+} for the activity, and effect of molybdate on the activity.

The *N*-terminal amino acid sequence of AP35, transferred from SDS-PAGE gel to a clear blot membrane-P (Atto) by electroblotting, was examined by use of an automatic protein sequencer (PPSQ-23, Shimadzu). The sequence of *N*-terminal 20 amino acid residues identified was Gly-Ile-Pro-Ala-Ala-Gly-Glu-Phe-Thr-Gln-Ala-Gln-Pro-Ile-Asn-Thr-Gly-Gly-Gly-Asp. A computer search found that the sequence has no significant similarity with protein sequences entered in the Swiss Prot and PIR Prot databases. The sequence also did not show similarity with the deduced amino acid sequence of the *agp* gene encoding Glc-1-phosphatase from *E. coli*.[24]

In this study, we first found an acid phosphatase with a high specificity against Glc-1-phosphate in mycelia of *P. nameko* but the physiological function remains unclear. Therefore, studies on the localization, the gene cloning, and the regulation of the expression by glucose or P_i are under way to obtain more detailed information of the enzyme.

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