Secretion of 112 kDa Phosphatase from the Cultured Suspension Cells of Liverworts

Ayako Nishizaki, Atushi Satake, Li Chen, Shunsuke Izumi, and Toshifumi Hirata*
Department of Chemistry, Faculty of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739

(Received June 12, 1996)

A phosphatase (homodimer of 56 kDa subunits) having a hydrolyzing activity toward *p*-nitrophenyl dihydrogenphosphate and phosphohistone was found to be secreted from cultured suspension cells of *Marchantia polymorpha* into their culture medium. Such secretion of the phosphatase was promoted by addition of bornyl acetate into the culture medium.

In general, higher plants are capable to respond with defense reactions against pathogenic fungi or bacteria.[1] One of these defense reactions of higher plants is the induction of certain hydrolytic enzymes, e.g. β-glucanase[2-4] and chitinase,[5] which dissolve the cell walls of pathogenic fungi. Furthermore, the secretion of peroxidase,[6] glucosidase,[7] ascorbate reductase[8] and esterase[9] from plant cell cultures has been also reported.

On the other hand, we found that the cultured cells of *Marchantia polymorpha* (liverworts) secrete allelochemicals when treated with bornyl acetate (**1**).[10] In connection with the studies on the defense reactions of plant cells, we now found that a phosphatase is secreted from the cultured cells of *M. polymorpha* by stimulation with bornyl acetate (**1**).

In order to clarify the secretion of phosphatase from the cultured cells of *M. polymorpha*,[11] the time course of the activity of phosphatase in the medium during incubation was followed. The cultured cells of *M. polymorpha* (10 g) were transplanted to 50 ml of freshly prepared MSK-2 medium[12] in 100 ml conical flasks and the cultures were incubated at 25 °C on a rotary shaker under illumination. For the stimulation experiments, bornyl acetate (**1**) was added to the suspension cells of *M. polymorpha* to a concentration of 1.3 mM. At a regular time interval, one of the cultures was filtered and the protein concentration and phosphatase activity[13] of the cultured medium were determined. The phosphatase activity kept increasing over a period of 7 days, as shown in Figure 1. When bornyl acetate (**1**) was added into the suspension cell cultures, the phosphatase activity jumped up to about 2-fold. This indicated that the cultured cells of *M. polymorpha* secreted a phosphatase into the cultured medium, and responded to bornyl acetate (**1**) with an increase in the secretion of phosphatase.

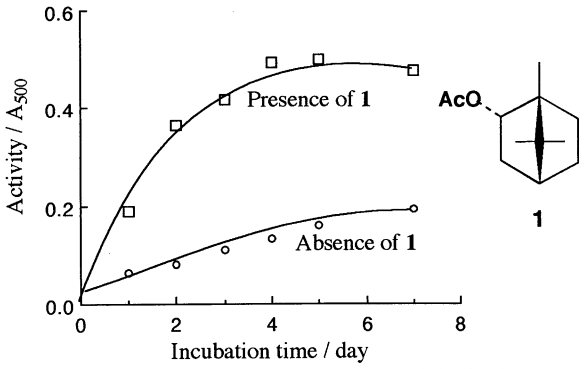

Figure 1. Changes in the phosphatase activity in the presence or the absence of bornyl acetate (**1**) in the cultured medium during growth of the suspension cells of *M. polymorpha*.

Table 1. Purification of the phosphatase from the cultured medium of *M. polymorpha*

Step	Total protein mg	Total act. μkat [a)]	Purification Fold
Ammonium Sulfate	18.0	1.51	1
Phenyl-Toyopearl	1.46	1.37	11
DEAE-Toyopearl	0.70	1.17	20
TSK gel DEAE-5PW	0.014	0.10	85

a) One kat was defined as the amount of enzyme which hydrolyzed 1 mol / s of *p*-nitorophenyl dihydrogenphosphate.

The phosphatase secreted from the cultured cells of *M. polymorpha* was isolated and purified. The cultured medium filtered from the suspension cultures (about 10 days old) was treated with ammonium sulfate. The protein precipitated with 40--80% saturation of ammonium sulfate was collected and dissolved with 10 ml of 10 mM Tris-HCl buffer, pH 7.0. The solution was subjected to a molecular sieve (Phenyl-TOYOPEARL) and a anion-exchange (DEAE-TOYOPEARL) columns, and then HPLC on TSKgel DEAE-5PW. In these purification processes, the phosphatase was eluted as one major peak and purified about 85 fold against the ammonium sulfate fraction, as shown in Table 1. The final preparation gave a single protein band corresponding to a molecular weight of 56 kDa on SDS-PAGE,[15] which is similar to the reported molecular weight of phosphatases from *Vigna sinensis* seeds[16] and sweet potato.[17] However, the MALDI-TOF mass spectrum of the purified phosphatase showed its molecular weight to be 112 kDa, as shown in Figure 2.[18] Thus the phosphatase was found to be a homodimer of 56 kDa subunits.

The phosphatase was maximally active at pH 6.5 and the activities at pH 5.4 and 8.5 were approximately 50% of the maximal activity. The protein was stable below 45 °C and was completely inactivated at 60 °C. Complete inhibition of the phosphatase activity was observed with 2 mM Hg^{2+} or Mo^{2+} and about 50% inhibition with V^{2+} and KH_2PO_4. On the other hand, the phosphatase activity was not inhibited by 2 mM KF. This suggests that the phosphatase may not be a kind of acid phosphatases, which were found in plants.[17,19,20]

The fact that the phosphatase was inhibited with Mo^{2+} and V^{2+} suggested the enzyme may be a protein phosphatase.[21] Therefore, the protein phosphatase activity was measured by using [^{32}P]phosphohistone[22] as substrate. By a 10-min incubation of [^{32}P]phosphohistone with the phosphatase, about 3% of the total radioactivity of [^{32}P]phosphohistone was found in the dissociated Pi, although the heated phosphatase was not active for the hydrolysis.

Thus it was demonstrated that phosphatase is secreted from the cultured suspension cells of *M. Polymorpha* in the medium during incubation. It was reported that phosphatase is

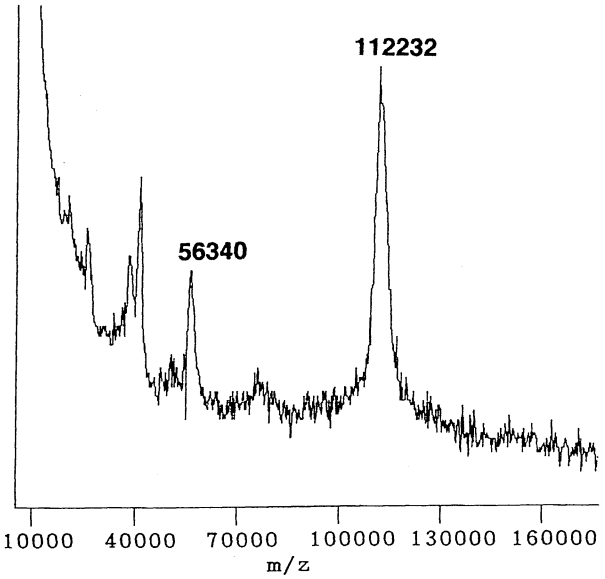

Figure 2. MALDI-TOF-mass spectrum of the phosphatase from the cultures of *M. polymorpha*.

secreted from other plant cells[24,25] and takes part in the rapid transformation of nucleotides into permeable compounds, which can be taken up by the cells and incorporate into DNA.[25] The phosphatase from cultured cells of *M. polymorpha* might act as a protein phosphatase responsible for the protection of the cells. However, which role the secreted phosphatase plays in the defense reactions of plants is not clear. Further works are necessary to clarify the physiological functions of the secreted phosphatase.

The authors thank the Application and Research Center, JEOL Ltd., for the measurement of the MALDI-TOF mass spectrum.

References and Notes

1 A. A. Bell, *Ann. Rev. Plant Physiol.*, **32**, 21 (1981).
2 F. B. Abeles, R. P. Bosshart, L. E. Forrence, and W. H. Habig, *Plant Physiol.*, **47**, 129 (1970).
3 P. M. Wargo, *Physiol. Plant Pathol.*, **5**, 99 (1975).
4 D. Nrtzer, G. Kritzman, and I. Chet, *Physiol. Plant Pathol.*, **14**, 47 (1979).
5 M. Esaka, A. Toyota, and H. Hayakuma, *Physiol. Plant.*, **92**, 90 (1994).
6 R. N. Chibbar, R. Cella, D. Albani, and R. B. V. Huystee, *J. Exp. Botany*, **35**, 1846 (1984).
7 Y. Yamazaki and H. Konno, *Agric. Biol. Chem.*, **49**, 3383 (1985).
8 M. Esaka and T. Asahi, *Plant Cell Physiol.*, **1982**, 315 .
9 S. Izumi, Y. Yamamoto, and T. Hirata, *Phytochemistry*, **38**, 831 (1995).
10 L. Chen, S. Izumi, D. I. Ito, T. Iwaeda, R. Utsumi, and T. Hirata, *Chem. Lett.*, **1996**, 205.
11 The suspension cultures of *M. polymorpha* were maintained by incubating 100 ml of fresh MSK-2 medium[12] containing about 2-5 g of *M. polymorpha* cells in 300 ml conical flasks at 25 °C on a rotary shaker at 70 rpm under 3000 lux illumination.
12 K. Ono, K. Ohyama, and O. L. Gamborg, *Plant Sci. Lett.*, **14**, 225 (1979).
13 According to the reported method,[14] the phosphatase activity was determined by measuring the A_{500} of a complex of Fe^{2+} and *p*-nitrophenol hydrolyzed from *p*-nitrophenyl dihydrogenphosphate.
14 O. Daniel, P. R. N. King, and E. J. King, *Brit. Med. J.*, **1954**, 19.
15 SDS-PAGE was run in a gel containing 12.5% (w/v) polyacrylamide and 0.1% (w/v) SDS. Prior to electrophoresis, the sample protein was heated at 90 °C for 30 min in 0.01 M Tris-HCl buffer soln (pH 6.8) containing 0.2% (w/v) of SDS, 1% (w/v) of 2-mercaptoethanol, and 2% (w/v) of glycerol.
16 T. K. Biswas and C. Cundiff, *Phytochemistry*, **30**, 2119 (1991).
17 K. Uehara, S. Fujimoto, T. Taniguchi, and K. Nakai, *J. Biochem.*, **75**, 639 (1974).
18 MALDI-TOF mass spectrum of the sample protein in 10 mM Tris-HCl buffer was recorded on a JMS-LDI 1700 instrument at MULDI(+) ionization mode (Repeller 30 kV and Extractor 10.5 kV) using sinapinic acid as matrix.
19 S. Izumi, Y. Ashida, T. Yamamitu, and T. Hirata, *Experientia*, **52**, 81 (1996).
20 S. Fujimoto, T. Nakagawa, and A. Ohara, *Chem. Pharm. Bull.*, **25**, 3283 (1977).
21 G. M. Polya and M. Haritou, *Biochem. J.*, **251**, 357 (1988).
22 [^{32}P]phosphohistone was prepared from dephosphorylated histone by the phosphorylation with [γ-^{32}P]ATP and protein kinase A.[21,23]
23 N. T. Tonks, C. D. Diltz, and E. H. Fischer, *J. Biol. Chem.*, **263**, 672 (1988).
24 K. Ueki and S. Sato, *Physiol. Plant.*, **24**, 506 (1971).
25 G. Ciarrochi, R. Cella, and E. Nielsen, *Physiol. Plant.*, **53**, 357 (1981).