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Short Communication

Electrophoretic analysis of soluble proteins specifically synthesized under phosphate deficiency in the mycelia of *Pholiota nameko*

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Mycelial soluble proteins of *Pholiota nameko* labeled in vivo during the Pi-supplied (P^+) and the Pi-depleted (P^-) cultures were separated by SDS-polyacrylamide gel electrophoresis and two-dimensional polyacrylamide gel electrophoresis, and visualized by fluorography. A comparison of protein profiles from the P^+ and P^- cultures showed that Pi deficiency induces the synthesis of 15 polypeptides and an increase in the relative amount of 29 polypeptides. These result suggests that many proteins may be specifically synthesized de novo under Pi deficiency as part of the adaptive mechanism for this condition.

Key Words——*Pholiota nameko*; phosphate deficiency; SDS-PAGE; soluble protein; two-dimensional-PAGE.

Phosphate is an essential nutrient for all organisms. Plants and microorganisms can incorporate phosphate only as inorganic phosphate (Pi). Therefore, phosphate is apt to be deficient for them. Most of them possess an adaptive mechanism against Pi deficiency, although there might be differences in their adaptive abilities. A representative example of the mechanism is an elevation of intracellular and extracellular acid phosphatase activities, as reported in various plants and microorganisms (Caddick and Arst, 1986; Duff et al., 1991; O'Connell and Grove, 1985; Schurr and Yagil, 1971; Tadano and Sakai, 1991; Ueki and Sato, 1971). Besides phosphatase, other enzyme activities have been reported to be altered by Pi deficiency (Duff et al., 1989; Li and Ashihara, 1990; Nagano and Ashihara, 1993; Smyth and Chevalier, 1984; Usuda and Shimogawara, 1991, 1992). Although a change in the protein profile must be involved in the adaptive processes, the overall picture remains unclear. We previously compared soluble mycelial proteins of *Pholiota nameko* (T. Ito) S. Ito et Imai apud Imai cultured in the Pi-supplied (P^+) and Pi-depleted (P^-) media by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and found that some protein bands were newly appeared or increased in the intensity specifically during the P^- culture (Joh et al., 1996b). In this study, to further investigate changes in mycelial soluble proteins resulting from Pi deficiency, proteins labeled in vivo during P^+ and P^- cultures were analyzed by SDS-PAGE and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

Mycelia of the strain N114 of *P. nameko* (Tohoku Shiitake) were cultured in the P^+ and P^- media containing phosphate at the concentrations of 550 and 50 mg/L as KH_2PO_4, respectively, as described previously (Joh et al., 1996b). On day 10 of cultivation, 150 μl of distilled water containing 3.33 MBq of [^{35}S] methionine (Methionine-35S-label, 37 TBq/mmol, American Radiolabeled Chemicals) was added to the culture fluid in a flask. After labeling for various periods, the mycelia were collected by centrifugation at 8,000 $\times g$ for 30 min at 4°C, washed three times with 0.2 M acetate buffer (pH 5.5), then homogenized in the buffer with a Polytron homogenizer (Model K; Kinematica) at about 13,000 rpm for 2 min at 4°C. The homogenate was centrifuged at 10,000 $\times g$ for 30 min at 4°C, and the supernatant was used for the following experiments.

Incorporation of [^{35}S] methionine into soluble mycelial protein during the P^+ and P^- cultures was determined by counting the radioactivity incorporated into the protein fraction precipitated with 10% trichroloacetic acid. Figure 1 shows the time course of incorporation. Following the addition of methionine on day 10 of cultivation, the incorporation per flask in the P^+ culture increased remarkably and reached the maximum level after 2 d, while that in the P^- culture increased gradually up to 4 d. The amount of incorporation in the P^+ culture was much higher than that of the P^- culture. We previously reported that the mycelial protein content per flask in the P^+ culture increased remarkably between 10 and 20 d of cultivation and was significantly higher than the corresponding content of the P^- culture, but no significant difference in the protein content on a dry weight basis was found between the two cultures during this period (Joh et al., 1996b). Therefore, the high incorporation in the P^+ culture is probably attributable not to the high pro-

tein content but to the high rate of the mycelial growth.

Proteins labeled in vivo for 4 d beginning on day 10 of cultivation were analyzed with SDS-PAGE by the method of Laemmli (1970). The soluble protein extracts from the P^+ and P^- cultures containing 2×10^5 cpm of radioactivity were each loaded on 12.5% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS and separated by electrophoresis at 30 mA for 3 h. The gel was fluorographed by treating with EN^{3}HANCE (Du Pont), dried, and exposed to Kodak XAR-5 film at −80°C. As shown in Fig. 2, obvious differences in the band patterns were observed between the P^+ and P^- cultures. Some specific bands with high relative intensity were detected in each pattern. This result suggests that the degradation of proteins is not the reason for the difference in the protein profile between the two cultures, and that specific proteins are synthesized de novo in the P^- culture.

The limited resolution afforded by SDS-PAGE makes it difficult to examine quantitative changes of many proteins. Therefore, the labeled proteins were next analyzed with 2D-PAGE by the method of O'Farrell (1975). Soluble proteins on the electrogram were resolved into more than 200 spots, of which about 50% were common to both cultures, about 30% were rich in the P^+ culture, and about 20% were rich in the P^- culture (Fig. 3). Because the final aim of the present study was to clarify the function of proteins relating to the adaptive mechanism for Pi deficiency, we only dealt with spots that were thicker in the P^- culture than the P^+ culture. Twenty-nine spots (A–Z and a–c) that were thicker in the P^- culture but visible in both cultures were detected. The intensity of the spots represented not the net amounts but the relative abundance of the corresponding polypeptides in the P^- culture. However, the result suggests that the polypeptides may increase specifically by Pi deficiency. Fifteen spots (1–15) were visible only in the P^- culture. Therefore, the corresponding polypeptides were regarded as being specifically induced by Pi deficiency.

In the 2D-PAGE analysis, 44 polypeptides that were synthesized in relative abundance in the P^- culture were detected, suggesting that the dynamic change in the protein synthesis may be caused by Pi deficiency in the mycelia of *P. nameko*. Usuda and Shimogawara (1995) examined the 2D-PAGE pattern of soluble protein from maize leaf during Pi deprivation and found 18 polypeptides that increased in relative abundance. Fife et al. (1990) identified four proteins in suspended cells of *Brassica nigra* (L.) Koch by 2D-PAGE whose de novo synthesis rates were increased under Pi-starved conditions. These show that many genes may be specifically expressed under Pi-deficient conditions and their corresponding proteins may participate in the adaptive mechanism. As described previously (Joh et al., 1996b), the mycelia of *P. nameko* seemed to grow well under Pi deficiency. The dynamic change in the protein synthesis of *P. nameko* may reflect its high adaptive capability for Pi deficiency.

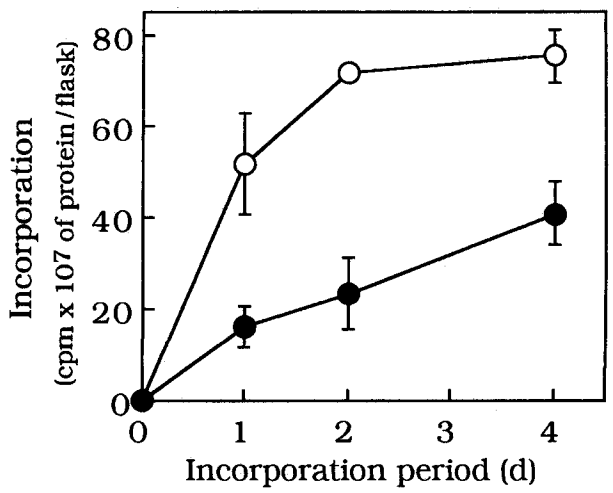

Fig. 1. Time course of incorporation of methionine into protein during the P^+ and P^- cultures.
[^{35}S] Methionine was added into the culture fluid on day 10 of cultivation and the subsequent incorporation into soluble mycelial protein was measured. Data are means and SE of three replicates. ○, P^+ culture; ●, P^- culture.

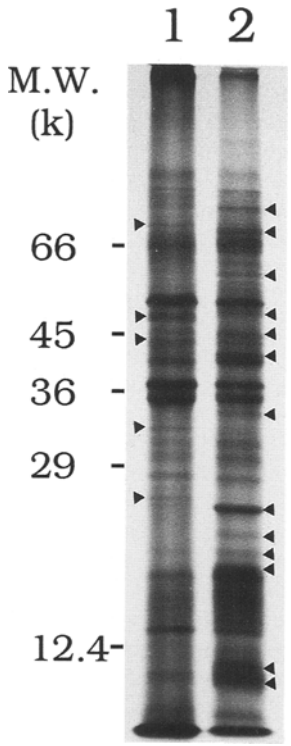

Fig. 2. SDS-PAGE analysis of in vivo labeled soluble mycelial proteins.
Lanes: 1, P^+ culture; 2, P^- culture. Numbers in the left margin indicate molecular weight of the marker proteins. Arrowheads on the left and right sides indicate intense bands in the P^+ and P^- cultures, respectively.

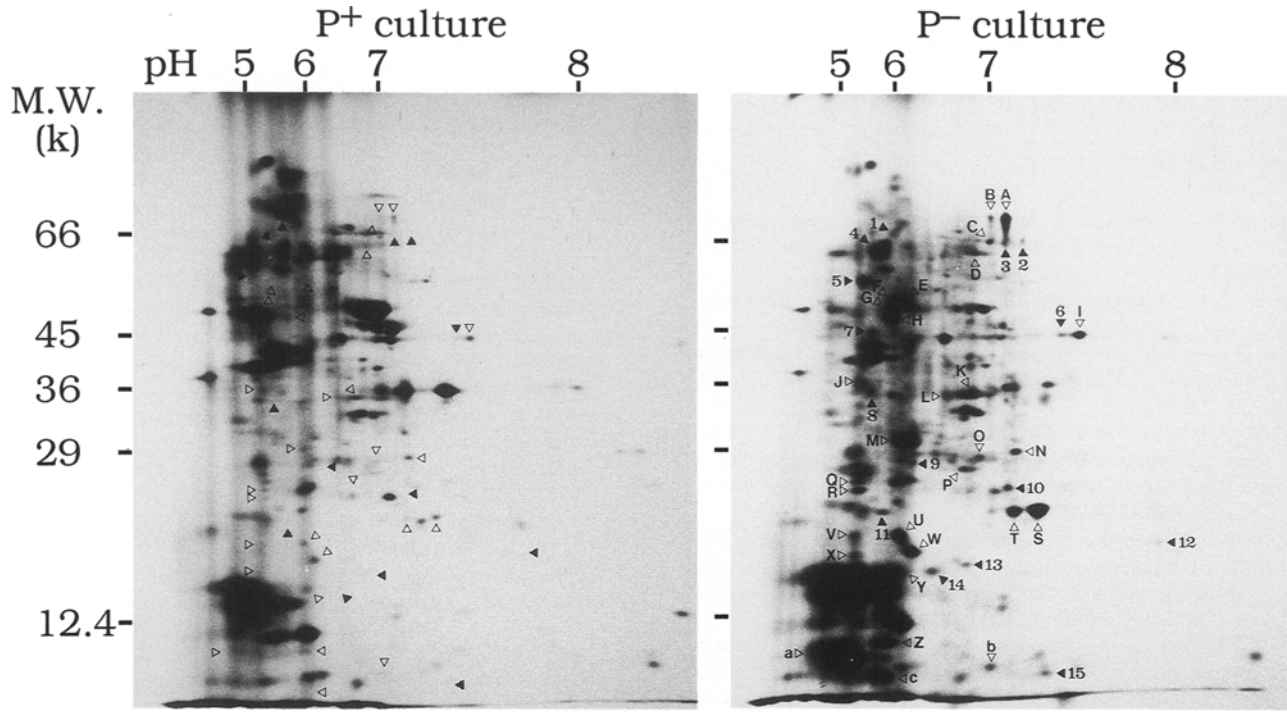

Fig. 3. 2D-PAGE analysis of in vivo labeled soluble protein from mycelia cultured in the P^+ and P^- media. Left panel, P^+ culture; right panel, P^- culture. Open and closed arrowheads indicate polypeptides that were increased in the relative amount and induced by Pi deficiency, respectively.

The induction of acid phosphatase by Pi deficiency has been reported in many organisms as described above. In the mycelia of *P. nameko*, three acid phosphatase isozymes with molecular weights of 47,000, 48,000 and 52,000 (APase47, APase48 and APase52, respectively) have been found to be induced and secreted by the deficiency (Joh et al., 1996a, b; Yazaki et al., 1997). Of these, only APase47 and APase48 are detectable by the activity staining after SDS-PAGE (Yazaki et al., 1997). In the 2D-PAGE analysis, two spots (G and H in Fig. 3) with the same molecular weights as APase47 and APase48 were detected as polypeptide specifically synthesized under Pi deficiency. However, no increase in intensity of a spot with molecular weight corresponding to APase52 was found, probably owing to overlap of spots. When the mycelial proteins of the P^+ and P^- cultures were activity-stained by the method of Lacks and Springhorn (1980) after 2D-PAGE, three active spots at the positions corresponding to spots F, G, and H were observed only in the P^- culture (Fig. 4). The result shows that three acid phosphatase isozymes, including one not hitherto found, are specifically synthesized in the P^- culture.

To our knowledge, in addition to phosphatase, only ribonuclease (Loffler et al., 1992), phosphoenolpyruvate carboxylase (Duff et al., 1989; Johnson et al., 1996) and pyrophosphate-dependent phosphofructokinase (Duff et al., 1989; Theodorou et al., 1992) have been identified among functional proteins induced by Pi deficiency. An

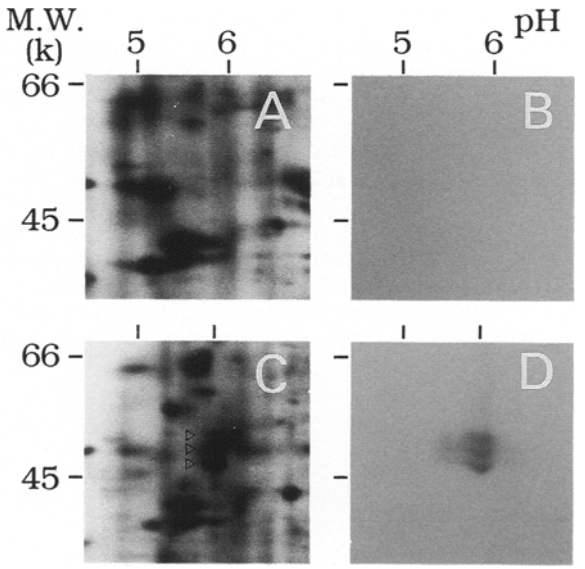

Fig. 4. Induction of acid phosphatase isozymes by Pi deficiency. Labeled soluble mycelial proteins from the P^+ (A and B) and P^- (C and D) cultures were separated by 2D-PAGE, then visualized by fluorography (A and C) or activity-staining for acid phosphatase (B and D). Arrowheads indicate spots corresponding to acid phosphatases.

attempt to estimate the functions of proteins identified in this study from their N-terminal amino acid sequences was unsuccessful, because the N-terminus of almost all proteins tested was modified.

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