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Specific inhibitor and substrate specificity of alkaline phosphatase expressed in the symbiotic phase of the arbuscular mycorrhizal fungus, *Glomus etunicatum*

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Abstract: Specific inhibitor and substrate specificity of alkaline phosphatase in the arbuscule of *Glomus etunicatum* were investigated, and the possible role of this enzyme in the symbiosis was discussed. Mycorrhizal roots of marigold (*Tagetes patula*) were digested by cellulase and pectinase to separate the intraradical hyphae from the root tissue, and phosphatase activity was stained at pH 8.5 and 5.0. The activity of alkaline phosphatase (pH 8.5) in arbuscules was inhibited in the presence of beryllium, whereas that of acid phosphatase (pH 5.0) was less sensitive to beryllium. Specificity and effectiveness of beryllium on the alkaline phosphatase was further confirmed using fractionated (soluble and insoluble) enzyme prepared from the separated hyphae. The soluble and insoluble alkaline phosphatases hydrolyzed phosphomonoester compounds (glucose-6-phosphate, β -glycerophosphate, trehalose-6-phosphate and glucose 1-phosphate) but not pyrophosphate compounds (ATP and polyphosphate) which were hydrolyzed by acid phosphatase efficiently. The insoluble alkaline phosphatase showed high specific activity (on a protein basis) and high sensitivity to beryllium. Kinetic analysis of the insoluble alkaline phosphatase suggested the involvement of this enzyme in the sugar metabolism of the fungus due to lower K_m values for sugar phosphate such as glucose-6-phosphate and trehalose-6-phosphate.

Key Words: beryllium, enzyme, sugar phosphate

INTRODUCTION

The arbuscule is a distinctive organ formed in arbuscular mycorrhizas. It is supposed that the arbuscular interface is the site of phosphate transfer from the fungus to the host, because H^+ -ATPase activity coupled phosphate uptake would be associated at the periarbuscular membrane (Gianinazzi-Pearson et al 1991, Smith and Read 1997). Alkaline phosphatase (ALPase) in arbuscular mycorrhizal fungi is an enzyme specific to the symbiotic status (Saito 1995) and localized at fine structural (mature) arbuscules (Ezawa et al 1995, Gianinazzi et al 1979), and this contrasts with acid phosphatase (ACPase) which was active both at the symbiotic and nonsymbiotic phases (Macdonald and Lewis 1978, Saito 1995). Because of the specific localization the involvement of ALPase in phosphate transfer has been suggested (Gianinazzi-Pearson and Gianinazzi 1983, Guillemin et al 1995, Tisserant et al 1993). The characteristics of ALPase, however, are not well understood, although it is essential to understanding the metabolic role of this enzyme.

Crude enzyme preparations or tissue of mycorrhizal roots were used in past studies on mycorrhizal phosphatases. However, several phosphatases, such as nonspecific phosphatases (Ezawa et al 1995, Gianinazzi et al 1979, Gianinazzi-Pearson and Gianinazzi 1978, Saito 1995), neutral phosphatase (Jeanmaire et al 1985), ATPases (Gianinazzi-Pearson et al 1991, McArthur and Knowles 1993) and polyphosphatases (Capaccio and Callow 1982), could be associated with those preparations, and this made it unclear which phosphatase would be responsible to the activity. A specific inhibitor is useful to identify the activity of specific enzyme. Cyanide (CN^-) was used as a specific inhibitor for the mycorrhizal alkaline phosphatase (Gianinazzi-Pearson and Gianinazzi 1978, Tisserant et al 1993) but did not inhibit the activity in the intact hyphae of *Glomus etunicatum*, *G. mosseae* and *Gigaspora rosea* (Ezawa et al 1995). Beryllium (Be^{2+}) is known as a specific inhibitor of animal and bacterial ALPases (Klemperer et al 1949, Thomas and Aldridge 1966) and 1000-fold as strong as those of metal chelators such as cyanide and EDTA (Bell 1972, Thomas and Aldridge 1966).

In this study, the intraradical hyphae of *Glomus etunicatum* were separated from the roots by the en-

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zymatic digestion technique, and specificity and effectiveness of Be^{2+} on the alkaline phosphatase in the hyphae were demonstrated. Furthermore, substrate specificity of the enzyme was investigated based upon the specific inhibition with Be^{2+} .

MATERIALS AND METHODS

Separation of intraradical hyphae.—Dwarf marigold (*Tagetes patula* cv. Bonanza Spray) was inoculated with *Glomus etunicatum* and cultured in a growth chamber as described by Ezawa and Yoshida (1994). Mycorrhizal roots were harvested 6 wk after sowing, cut into 5–10 mm segments, incubated at 30 °C for 60 min in a 10-fold vol (w:vol) of 20 g L⁻¹ Cellulase Onozuka RS (Yakult Pharmaceutical Ind., Tokyo), 20 g L⁻¹ Driselase (Kyowa Hakko Kogyo, Tokyo) and 10 g L⁻¹ Pectolyase Y-23 (Seishin Corp., Tokyo) in 10 mM MES/NaOH (pH 5.5) and washed in washing buffer (1 mM dithiothreitol, 0.3 M mannitol, 10 mM Tris/HCl, pH 7.6) at 0 °C (Ezawa et al 1995). The root cortex became translucent at this step. And colonized roots were selected under a dissecting microscope.

Histochemical study.—The cortex of digested roots was torn with needles to expose the fungal tissue. Phosphatase activity in the hyphae was visualized by the azo dye method (Scandalios 1969). The fungal tissue was preincubated for 30 min at 35 °C in the preincubation solution (100 mM Tris/HCl buffer pH 8.5 or 100 mM acetate buffer pH 5.0) with or without 1 mM BeSO_4 , then transferred to the staining medium (4 mM α -naphthyl acid phosphate (substrate), 1 g L⁻¹ Fast Blue RR salt (azo dye) in 100 mM Tris/HCl buffer or 100 mM acetate buffer) with or without 1 mM BeSO_4 and incubated at 35 °C for 120 min.

After staining, the specimens were transferred to lactoglycerol and mounted on glass slides. The numbers of arbuscules showing phosphatase activity (indicated by black precipitation) per unit length (200 μm) of colonized root were measured at $\times 100$ –200. The measurements were made at five different positions per root segment, and at least 10 segments were observed for each treatment.

Fractionation of hyphal enzyme.—Intraradical hyphae were collected from the digested roots with fine needles under a dissecting microscope, weighed and stored in a 1.5 mL microtube at -85 °C.

Two to four mg (FW) of the fungal tissue was ground in a small mortar (6 cm diam) with 10-fold vol (w:vol) of the extraction buffer (0.88 M sucrose in 100 mM Tris/HCl pH 7.5) at 0 °C. The slurry was transferred to a 1.5-mL microtube. The mortar and pestle were washed with the same vol of extraction buffer. The solutions were combined and centrifuged at 10 000 g for 10 min at 4 °C, and the residue was resuspended in a 20-fold vol of the buffer and centrifuged. The supernatants were combined and the volume was adjusted to 100-fold vol of the original weight with the buffer (soluble fraction). The residue was washed twice in the buffer and suspended in 100-fold vol of the buffer (insoluble fraction).

TABLE I. Influence of beryllium on the frequency of arbuscules showing activity of phosphatase of *Glomus etunicatum*

pH	Active arbuscules/200 μm	
	Control	1 mM BeSO_4
8.5	12.3 $\pm$ 1.8 ^a (100) ^b	0.2 $\pm$ 0.1 (1.6)
5.0	9.8 $\pm$ 1.4 (100)	7.1 $\pm$ 0.4 (72.5)

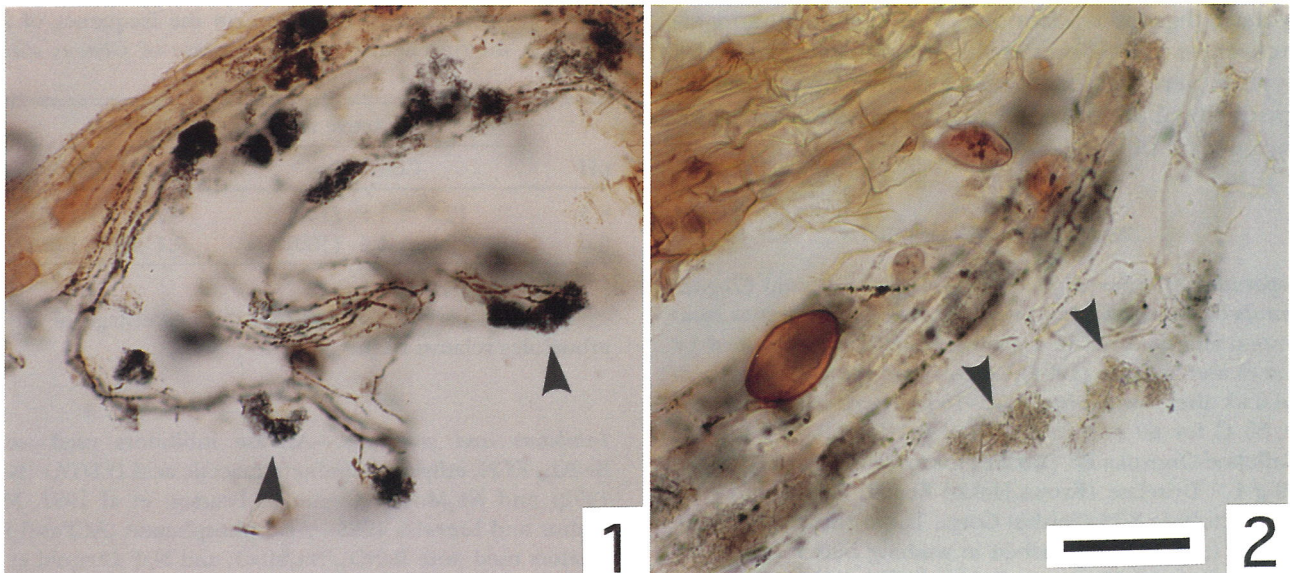
^a $\pm$ SE (n = 10)

^b Values in parentheses indicate the percentage of active arbuscules relative to the control.

Inhibitors and substrates.—ALPase inhibitors used were BeSO_4 , KCN, ethylenediaminetetraacetic acid (EDTA) (Bell 1972) and Na_2MoO_4 (Gianinazzi-Pearson et al 1991, McArthur and Knowles 1993). Acid phosphatase (ACPase) inhibitors used were BeSO_4 , Na_2MoO_4 and NaF (Arnold et al 1987). All these inhibitors were purchased from Wako Pure Chemical, Japan.

All substrates for phosphatase assays were purchased from Sigma Chemical Co. Polyphosphate ranging in chain-length from 90 to 190 was prepared by electrophoresis as described by Clark and Wood (1987). Polyphosphate Type 75+ was dissolved in electrophoresis buffer and loaded on a 10% polyacrylamide gel. After electrophoresis, the gel between the two tracking dyes, bromophenol blue and xylene cyanol, was cut and polyphosphate in the gel piece was extracted and concentrated. Polyphosphate in the extraradical hyphae of *G. etunicatum* was extracted, treated with ribonuclease (Wako Pure Chemical, Japan) for 30 min at 55 °C and electrophoresed (Clark et al 1986) to check the suitability of the prepared polyphosphate as substrate for the fungal enzyme. The authors considered the prepared polyphosphate to be an appropriate substrate for the assessment of polyphosphate-hydrolyzing activity in the fungus, because the range of chain length of fungal polyphosphate was much broader than that of the prepared polyphosphate.

Enzyme assay conditions.—Extract (20–40 μL containing the soluble or insoluble enzyme from 0.2–0.4 mg hyphae) and substrate were incubated in 100 mM Tris/HCl buffer pH 8.5 or 100 mM acetate buffer pH 5.0 with or without phosphatase inhibitor in 400 μL total vol at 35 °C for 90 min [for *p*-nitrophenylphosphate (NPP) as substrate] or 120 min (for other substrate). After the incubation, the reaction mixtures of insoluble fraction were centrifuged immediately, and 360 μL of supernatant was transferred to a new tube. Insoluble materials in the tube were reserved to measure protein concentration. The reaction was terminated by adding 0.25 vol of 1 M NaOH (final concentration was 0.2 M, in the case of NPP) or a 0.67 vol of $\text{FeSO}_4(\text{NH}_4)_2\text{MoO}_4$ reagent (in the case of other substrate) (Taussky and Shorr 1953). The activity of phosphatase was determined by the absorbance of *p*-nitrophenol at 410 nm or the amount of inorganic phosphate released (Taussky and Shorr 1953). Under these conditions, the formation of product showed a linear relation with time. Protein concentration in the soluble fraction was determined by the method of Bradford



FIGS. 1, 2. Inhibition of alkaline phosphatase activity in the arbuscules (arrow heads) of *Glomus etunicatum* by beryllium. Phosphatase activity was indicated by black precipitation. 1. Control. 2. Stained in the presence of 1 mM BeSO_4 . Bar = 40 μm .

(1976). Protein concentration in the insoluble fraction was determined as follows. Insoluble materials remaining in the reaction tubes were combined (10–15 tubes into one), and an equal vol of 10% trichloroacetic acid was added. After incubation for 30 min at 4 C, the mixture was centrifuged,

and the supernatant was discarded. Protein in the residue was extracted by 50 μL of 1 N NaOH and quantified as described by Lowry et al (1951) because the method of Bradford (1976) could not be applied to the alkaline samples. One unit (U) of phosphatase activity was defined as the amount of enzyme which released 1 nmol of orthophosphate per min under the specific conditions. Kinetic parameters were determined by the statistical method described by Wilkinson (1961).

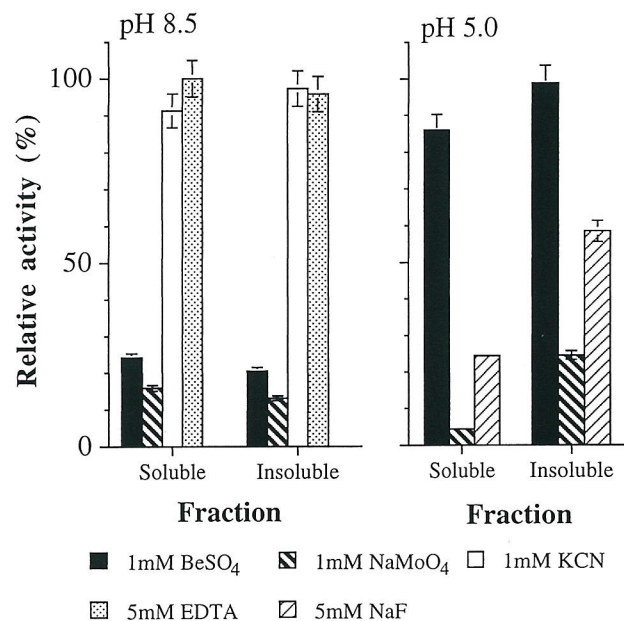


FIG. 3. Effect of various inhibitors on the relative activities of alkaline (pH 8.5) and acid (pH 5.0) phosphatases in the soluble and insoluble fractions of the intraradical hyphae of *Glomus etunicatum*. Phosphatase activities are expressed by percentage relative to the control (100%). The activity was measured by the absorbance of *p*-nitrophenol at 410 nm using 20 μL enzyme solution and 4 mM *p*-nitrophenylphosphate as a substrate. Vertical bars mean SE ($n = 3$).

RESULTS

Specific inhibition.—The inhibitory effect of Be^{2+} on phosphatase activities in arbuscules was examined at pH 8.5 (ALPase) and 5.0 (ACPase) histochemically and found to be specific to ALPase. The number of arbuscules showing phosphatase activity was greatly reduced in the presence of Be^{2+} at pH 8.5 (TABLE I, FIGS. 1, 2). In contrast, the effect of Be^{2+} on the activity in arbuscules at pH 5.0 was limited (TABLE I).

The effects of various inhibitors on the activity of fractionated enzymes at pH 8.5 and 5.0 were compared using NPP as substrate, and the effectiveness and specificity of Be^{2+} on the activity of ALPase was confirmed. Be^{2+} and molybdate (MoO_4^{2-}) inhibited the activity in both the fractions strongly at pH 8.5 (FIG. 3), but the metal chelators, EDTA and CN^- , did not affect the activities. Be^{2+} showed no inhibitory effect on the activity at pH 5.0, whereas strong inhibition by MoO_4^{2-} and moderate inhibition by fluoride (F^-) were observed at this pH. Based upon these observations, the authors defined the arbuscular ALPase as beryllium-sensitive activity at pH 8.5, thereafter.

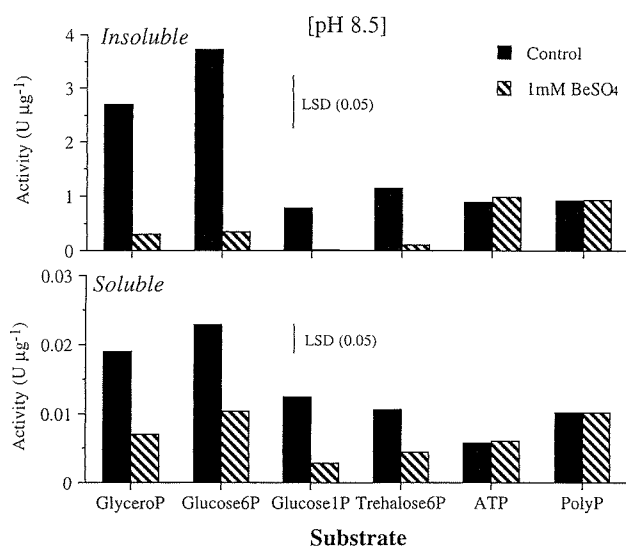


FIG. 4. Alkaline phosphatase activity (pH 8.5) of the insoluble and soluble fractions with various substrate in the presence or absence of 1 mM BeSO_4 . The concentration of substrate was 12 mM. GlyceroP, β -glycerophosphate; Glucose6P, glucose-6-phosphate; Glucose1P, glucose-1-phosphate; Trehalose6P, trehalose-6-phosphate; ATP, adenosine-triphosphate; PolyP, polyphosphate.

Substrate specificity.—Rates of hydrolysis of some natural phosphate compounds by ALPase were investigated, being compared with those by ACPase. Glucose-6-phosphate and β -glycerophosphate were hydrolyzed most efficiently by both the soluble and insoluble enzymes at pH 8.5 (FIG. 4). Glucose-1-phosphate, trehalose-6-phosphate, ATP and polyphosphate were also hydrolyzed. The activities with phosphomonoester compounds (glucose-6-phosphate, β -glycerophosphate, glucose-1-phosphate and trehalose-6-phosphate) were inhibited by Be^{2+} , but those with pyrophosphate compounds (ATP and polyphosphate) were not. The sensitivity of the insoluble enzyme to Be^{2+} was higher (80–90% inhibition) than that of the soluble enzyme (55–77% inhibition). The specific activity in the insoluble fraction was 100-fold higher than in the soluble fraction. Substrate specificity of the ACPases (pH 5.0) was different from that of the ALPase (FIG. 5). The insoluble enzyme showed a broad substrate specificity although ATP was less preferable. The soluble enzyme hydrolyzed pyrophosphate compounds more efficiently than phosphomonoester compounds.

Because a large part of ALPase activity was associated with the insoluble fraction, kinetic parameters of the insoluble ALPase with some phosphomonoester compounds were determined (TABLE II). The K_m values with glucose-6-phosphate and trehalose-6-phosphate (1.49 and 1.47 mM, respectively) were significantly lower than that of β -glycerophosphate

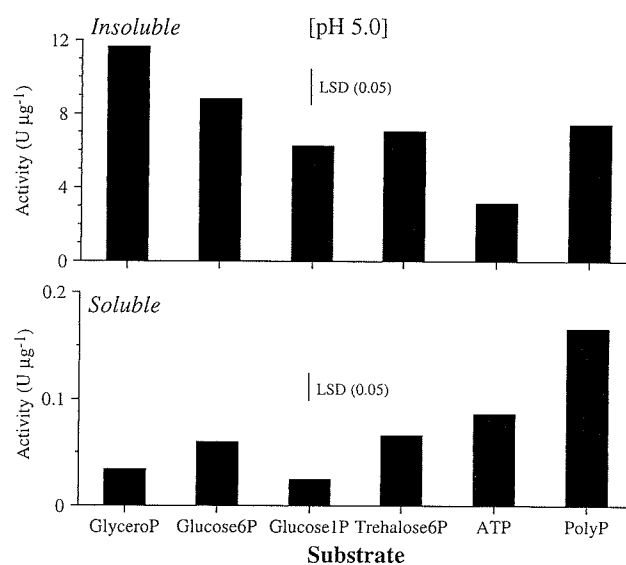


FIG. 5. Acid phosphatase activity (pH 5.0) of the insoluble and soluble fractions with various substrate. The concentration of substrate was 12 mM. The abbreviations of substrate were the same as FIG. 4.

(3.35 mM). The $V_{\max}$ values with glucose-6-phosphate (4.10 U μg^{-1}) and β -glycerophosphate (3.91 U μg^{-1}) were significantly higher than those of glucose-1-phosphate and trehalose-6-phosphate (1.03 and 1.32 U μg^{-1} , respectively).

DISCUSSION

This is the first report demonstrating the characteristics of mycorrhizal ALPase extracted directly from the pure fungal tissue. Characterization of the mycorrhizal ALPase has been reported for the soluble fraction extracted from colonized root tissue based upon electrophoretic analyses (Fabig et al 1989, Gianinazzi-Pearson and Gianinazzi 1976, 1978). It was uncertain, however, if the ALPase originated

TABLE II. Kinetic parameters of alkaline phosphatase in the insoluble fraction of the intraradical hyphae of *Glomus etunicatum*

Substrate	K_m (mM) ^a	$V_{\max}$ (U μg protein ⁻¹) ^a
β -glycerophosphate	3.35 $\pm$ 0.30 ^b	3.91 $\pm$ 0.94
glucose-6-phosphate	1.49 $\pm$ 0.72	4.10 $\pm$ 0.68
glucose-1-phosphate	2.22 $\pm$ 1.02	1.03 $\pm$ 0.34
trehalose-6-phosphate	1.47 $\pm$ 0.55	1.32 $\pm$ 0.26

^a Kinetic parameters were determined at pH 8.5 using a 40 μL enzyme solution (corresponding to 0.096 μg protein).

^b $\pm$ SE.

from the host or the fungus in these studies. Recently, a soluble fungal phosphatase from mycorrhizal roots was found to be of intraradical hyphal origin (Kojima et al 1998), but the enzyme has not yet been characterized. The present results also showed that considerable activity of the fungal ALPase was associated with the insoluble fraction. The authors considered that the insoluble ALPase might represent the activity revealed by the histochemical experiment because of the high specific activity (protein basis) and the high sensitivity to beryllium.

The histochemical and biochemical studies showed that the phosphatase activity at pH 8.5 in the arbuscules was inhibited strongly by beryllium, a typical characteristic of nonspecific alkaline phosphatase (ALPase) (E.C. 3.1.3.1) (Klemperer et al 1949, Thomas and Aldridge 1966). Lack of inhibition of acid phosphatase (ACPase) by beryllium was consistent with the observation of Klemperer et al (1949). This indicates that beryllium is a specific inhibitor for ALPase in the arbuscule, and further implies that the activity of this enzyme can be specifically determined in the crude extract containing other phosphatases by using beryllium. As shown by our previous study and the present result, we could not observe an inhibitory effect of cyanide. This is not consistent with the histochemical observations by Gianinazzi-Pearson and Gianinazzi (1978) and Tisserant et al (1993) in which different fungal species were used. Sensitivity of the ALPase of those fungi to cyanide should be confirmed by using enzyme preparations directly extracted from the pure fungal tissue.

The ALPase, unlike the ACPase, showed apparent substrate specificity. Glucose-6-phosphate showed the lowest K_m and highest V_{max} , indicating that this compound was the best substrate among the tested compounds for the arbuscular ALPase. Trehalose-6-phosphate would also be a good substrate due to a low K_m as that of glucose-6-phosphate. β -Glycerophosphate was hydrolyzed efficiently at higher concentration (8–12 mM), but the hydrolysis rate decreased considerably below 4 mM (data not shown) as reflected in the highest K_m (TABLE II). The arbuscular ALPase did not hydrolyze pyrophosphate compounds, such as ATP and polyphosphate. Soluble and insoluble (membrane bound) ALPases from *Bacillus licheniformis* also showed low activity with ATP (Hansa et al 1981), whereas extracellular ALPase from *Escherichia coli* (Reid and Wilson 1971) and *Neurospora crassa* (Say et al 1996) hydrolyzed ATP. Difference in catalytic activity between intra- and extracellular ALPases could be an indication of functional differentiation. Higher rate of hydrolysis of polyphosphate at pH 5.0 by the soluble enzyme was observed. Not only ACPase but also exopolyphosphatase

might be involved (Capaccio and Callow 1982, Ezawa and Saito unpubl).

In the symbiotic phase, the intraradical hyphae of arbuscular mycorrhizal fungi may take up glucose as an energy source (Shachar-Hill et al 1995, Solaiman and Saito 1997). Glucose-6-phosphate can be generated by the phosphorylation of glucose by hexokinase (Saito 1995) and/or polyphosphateglucokinase (Capaccio and Callow 1982, Ezawa and Saito unpubl) in the hyphae. Glucose-6-phosphate itself is a key compound of many important metabolic pathways in the fungi such as the pentose monophosphate cycle (Saito 1995) and the biosynthesis of trehalose (Schubert et al 1992, Shachar-Hill et al 1995) and glycogen (Shachar-Hill et al 1995). Trehalose-6-phosphate, an intermediary metabolite of trehalose biosynthesis, should be synthesized by trehalose-6-phosphate synthase from glucose-6-phosphate and UDP-glucose. It is suggested that the ALPase may be involved in the metabolism of these sugar phosphates.

Tisserant et al (1993) used ALPase activity as an index of phosphate translocation from the fungus to the host, suggesting a function of the ALPase such as a membrane transporter of inorganic phosphate. Conversely, Larsen et al (1996) found no correlation between phosphate translocation of the fungus and ALPase activity, because fungicide (benomyl) application inhibited fungal phosphate translocation but not ALPase activity in the hyphae. As they pointed out, however, the benomyl-mediated inhibition occurred in the extraradical hyphae which was far from the arbuscular interface. Assuming the possible role of the ALPase in the phosphate transfer, it is hypothesized that a part of phosphate released into the interfacial apoplast may be provided by the ALPase via the hydrolysis of intermediary metabolites such as sugar phosphates. Further studies focused on carbon and (poly)phosphate metabolism are required to examine this hypothesis.

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