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Alkaline Phosphatase and Other Hydrolyases Produced by *Cenococcum graniforme*, an Ectomycorrhizal Fungus

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Cell extracts of *Cenococcum graniforme* have been found to contain the following hydrolytic enzymes: protease, esterase, α-D-galactopyranosidase, β-D-galactopyranosidase, α-D-mannopyranosidase, β-D-xylopyranosidase, α-D-glucopyranosidase, β-D-glucopyranosidase, and alkaline phosphatase. Sulfatase, inorganic pyrophosphatase, and β-D-mannopyranosidase were not detected in the extracts. β-D-Xylopyranosidase and α-D-mannopyranosidase were most active in the neutral pH range, protease and phosphatase were most active in the alkaline pH range, and other enzymes were most active in the acidic pH range. These enzymes showed a high association with cell wall material, and the release of enzymes from the cells into the culture fluid appeared to occur only when the cells were undergoing autolysis. Alkaline phosphatase in *C. graniforme* is a constitutive enzyme, and examination of the alkaline phosphatase following a purification of 265-fold produced the following characteristics: pH optimum of 9.5, M_r of 60,000, K_m of 2.1×10^{-4} M for *p*-nitrophenylphosphate, and activation energy for hydrolysis of the substrate at 9.9 kcal (1 cal = 4.184 J)/mol.

The interaction between fungi and plant roots in the mycorrhizal state requires coordination of enzymatic activities between both living systems. Although the anatomical features of the mycorrhizae (27) and the identity of fungal partners (39) have received considerable attention, the enzymes associated with complex-carbohydrate utilization and the characteristics of enzymes purified from mycorrhizal fungi have not been elucidated. An important dimension of mycorrhizal activity is the uptake of phosphorus from the environment (3, 7, 34). Acid phosphatase activity has been demonstrated at the surface of roots containing ectomycorrhizal fungi (1, 4, 37) in vesicular-arbuscular mycorrhizal fungi (26, 36) and in ectomycorrhizal fungi maintained in axenic cultures (2, 22). The predominant form of phosphorus in the soil is organic phosphate (8), and the wide distribution of acid phosphatase in the ectomycorrhizal fungi would account for phosphorus nutrition in acidic environments. However, in alkaline soils, the acid phosphatases of the ectomycorrhizal fungi would not be functioning, and an alternate surface enzyme would be needed. Since aquatic and terrestrial fungi typically produce alkaline phosphatase (9, 11, 12, 23, 30, 38), we wished to explore alkaline phosphatase production by *Cenococcum graniforme*, a fungus frequently studied to determine nutrient mobilization and absorption potential of the mycorrhiza (10, 18, 29, 33).

Additionally, the problem of production of exocellular hydrolytic enzymes by ectomycorrhizal fungi is unresolved (19), since the demonstration of soluble exocellular enzymes could have resulted from cell autolysis and not from secretion of enzymes into the medium during growth. Ectomycorrhizae have been shown to produce phytase (35) and polyphenyl oxidases (17), which would be important in litter decomposition (15). In an attempt to understand the localization of hydrolytic enzymes produced by the ectomycorrhizal fungi, we explored the presence of exocellular, cell-bound, and intracellular hydrolytic enzymes in *C. graniforme*. We report the presence in *C. graniforme* of several enzymes which hydrolyze nitrophenyl (NP) substrates, starch, or casein, and we provide the first characterization of alkaline phosphatase purified from a mycorrhizal fungus.

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MATERIALS AND METHODS

Fungal growth. *C. graniforme* M-347 was cultivated in the medium described by Mexel and Reid (29), as modified by Rodriguez et al. (33). Evaluation of nutrients utilized by this fungus was accomplished by substitution of 1% carbon sources for glucose in the growth medium. Erlenmeyer flasks (150 ml) which contained 50 ml of autoclaved medium were inoculated with fungal mycelium and incubated at 20°C under stationary conditions. In order to achieve uniform inoculation, it was necessary to suspend mycelium by using a glass Ten Broeck tissue homogenizer with a clearance of 0.15 mm. Cell inocula, consisting of 2 mg (dry weight) of mycelium, were reproduced readily. After 30 days of growth, the mycelium was collected on Whatman no. 1 filter paper (Whatman Inc., Clifton, N.J.) and dried for 12 h at 80°C. Each growth response experiment was conducted in triplicate, and average values are presented here.

Cell disruption. For enzyme measurements, the fungal cells were grown for 30 days in 100 ml of media in 250-ml Erlenmeyer flasks which were shaken at 100 rpm. The carbon source was 1% glucose, and the phosphorus content resulted from the addition of 0.05% KH_2PO_4 and 0.025% K_2HPO_4. The mycelium was collected on filter paper and suspended in 0.01 M Tris hydrochloride (pH 8.0) with the glass Ten Broeck homogenizer. After one passage through the French pressure cell at 10,000 lb/in^2, the extract was subjected to centrifugation at 10,000 × *g* for 20 min at 5°C. The resulting supernatant was employed in the enzyme assays.

Enzyme measurements. Protease was measured by the casein digestion procedure described by McConn et al. (28). A unit of protease activity was described as the amount of enzyme which liberated 1 μg of 275-nm-light-absorbing material, calculated as tyrosine, in 1 h at 30°C.

Hydrolysis of *p*-NP-phosphate was measured by the procedure of Cohen (9). The 1.0-ml assays contained dialyzed cell extract from disrupted cells, 50 mM buffer unless specified, and 0.8 mM *p*-NP-phosphate. Buffers for alkaline, neutral, and acidic incubations consisted of Na_2CO_3-hydrochloride, Tris hydrochloride, and trisodium citrate-citric acid, respectively. To measure the hydrolytic activities of other enzymes, desired NP compounds at 0.8 mM were substituted for *p*-NP-phosphate in the reaction described above. The activities of the enzymes were detected by measuring the release of nitrophenol from the appropriate substrate. The enzymes and substrates, respectively, were as follows: esterase and *p*-NP-acetate; α-D-galactopyranosidase and *p*-NP-α-D-galactopyranoside; β-D-galactopyranosidase and *o*-NP-β-D-galactopyranoside; α-D-glucopyranosidase and *p*-NP-α-D-glucopyranoside; β-D-glucopyranosidase and *p*-NP-β-D-glucopyranosidase; α-D-mannopyranosidase and *p*-NP-α-D-mannopyranoside; β-D-mannopyranosidase and *p*-NP-β-D-mannopyranoside; β-D-xylopyranosidase and *p*-NP-β-D-xylopyranoside; and sulfatase and *p*-NP-sulfate. The molar extinction coefficients used to calculate the release of *p*-nitrophenol and *o*-nitrophenol were 1.62×10^4 at 410 nm and 1.8×10^4 at 420 nm, respectively. All NP compounds were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Inorganic pyrophosphatase was measured by the procedure of Koboyashi et al. (24). Tests for glucoamylase, α-amylase, and transglycosylase were conducted as previously described (5, 6, 31). Protein was determined by the use of the phenol reagent (25), and glucosamine measurements for the fungal cell wall were made by the procedure of Hepper (20).

Exocellular enzyme measurements. The culture filtrate was collected by passing *C. graniforme* cultures through Whatman no. 1 filter paper. The filtrate from a 30-day culture reflected the exocellular production of enzymes by a growing culture, while the filtrate from a 45-day culture represented the soluble enzyme following cell autolysis. The two samples of the culture filtrate were assayed for enzymes which would hydrolyze NP compounds, glucoamylase, α-amylase, and transglycosylase.

Purification of alkaline phosphatase. The procedure for purification of alkaline phosphatase was a modification of that employed for *Phycomyces blakesleeanus* (9). The cell extract was dialyzed overnight against 2.5 mM Tris hydrochloride (pH 8.0) at 4°C before it was adjusted to 80% saturation with $(NH_4)_2SO_4$. The precipitated protein was collected by centrifugation at $10,000 \times g$ for 10 min and suspended overnight against 2.5 mM Tris hydrochloride buffer (pH 8.0) before it was applied to a DEAE-cellulose column (2.5 by 18 cm) that had been equilibrated with 2.5 mM Tris hydrochloride (pH 8.0) buffer. A 0 to 1 M linear gradient of NaCl in the equilibrating buffer was used to elute the enzyme. The fractions containing the alkaline phosphatase were pooled and applied to a Sephadex G-150 column (1.8 by 25 cm). The buffer for column equilibration and elution was 2.5 mM Tris hydrochloride (pH 8.0).

Determination of M_r for alkaline phosphatase. Molecular weight measurements were determined from elution profiles from the Sephadex G-150 column on the basis of the procedure of Cohen (9). The void volume was established with blue dextran, while alcohol dehydrogenase (M_r, 150,000), bovine albumin (M_r, 66,000), carbonic anhydrase (M_r, 29,000), and cytochrome *c* (M_r, 12,400) were used as reference proteins. Enzymes for molecular weight determination and blue dextran were obtained from Sigma.

TABLE 1. Growth of *C. graniforme* on different carbon and nitrogen sources

Carbohydrate	Final pH	Cell mass (mg [dry wt]/50 ml)
Glucose	6.29	22.5 ± 2.0
Maltose	6.72	21.3 ± 1.6
Starch	6.40	12.4 ± 0.9
Sucrose	6.81	17.7 ± 1.0
None	6.57	2.0 ± .1

Regulation of alkaline phosphatase. To determine if the alkaline phosphatase in *C. graniforme* was subject to repression, the level of P_i in the medium was varied, and the resulting amount of enzyme was determined. Assays were performed on cell extracts from cultures grown for 30 days in 100 mm of medium.

RESULTS

Growth. Growth of *C. graniforme* on maltose was comparable to growth on glucose, while starch and sucrose were only slightly less effective as carbon and energy sources (Table 1). These growth responses were in agreement with those in a previous report (18). Starch utilization is usually attributed to exocellular enzyme production; however, we were unable to detect α-amylase, glucoamylase, or transglycosylase activity in the filtrate of the culture growing for 30 days with starch as the carbon source. In assays with mycelium, α-amylase, but not glucoamylase or transglycosylase, was detected, indicating that α-amylase was bound to the cell surface of the fungus.

In all cases of growth, *C. graniforme* grew as a black mycelial mass, and no pigment was released into the culture fluid in the first 30 days of growth. Continued incubation resulted in the release of a dark pigment from the mycelia, and by 45 days, extensive fragmentation of the mycelium was evident, resulting in a turbid blackish culture filtrate.

Enzymes in cell extracts. Protease activity was observed in cell extracts of *C. graniforme*, with the greatest activity at pH 9.0 but substantial activity at pH 6.0, suggestive of an acid protease (Fig. 1). Protease activity was bound to cell structures, since all activity remained in the black-pigmented cell material at the top of the DEAE-cellulose column and could not be eluted with changes of pH or salt concentrations. The dark material on the top of the column contained *N*-acetylglucosamine, presumably of cell wall origin.

Several enzymes which could hydrolyze NP substrates were present in the cell extract. Their activities are listed in Table 2. Assays conducted at pHs of 4.5, 7.0, and 9.5 revealed that enzymes which catalyzed the release of nitrophenol from *p*-NP-acetate, *o*-NP-β-D-galactopyranoside, *p*-NP-β-D-glucopyranoside, and *p*-NP-α-D-glucopyranoside had acidic pH optima. The enzyme hydrolyzing *p*-NP-phosphate was alkaline, and enzymes active on *p*-NP-α-D-mannopyranoside and *p*-NP-β-D-xylopyranoside had neutral pH optima. No enzymes which could hydrolyze *p*-NP-β-D-mannopyranoside or *p*-NP-sulfate were detected. The activities of cell extracts which hydrolyzed *p*-NP-α-D-mannopyranoside had pH optima of 6.0 (Fig. 2). There was no evidence for an inorganic pyrophosphatase in either soluble or cell-associated material in cell extracts from *C. graniforme*.

Alkaline phosphatase activity was present in cell extracts at levels greater than those of enzymes which hydrolyzed

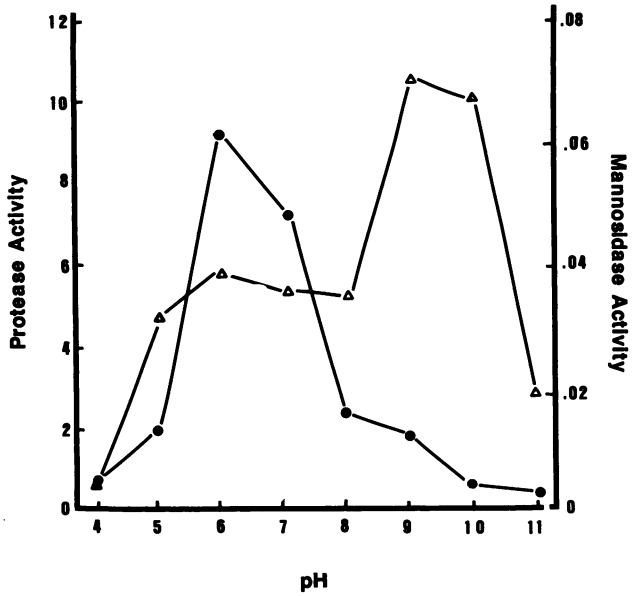

FIG. 1. Effect of pH on protease and mannosidase activities in extracts of *C. graniforme*. Protease activity (△) is given as micrograms of tyrosine released from casein per 60 min per mg of protein. Mannosidase activity (●) is given as micromoles of *p*-nitrophenol released from *p*-NP-α-D-mannopyranoside per 60 min per mg of protein.

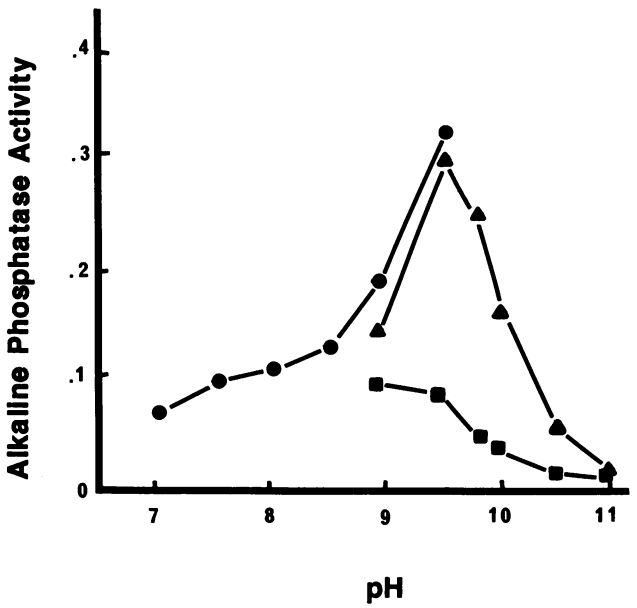

FIG. 2. Activity of alkaline phosphatase from *C. graniforme* at various pH levels. Buffer systems employed were Tris hydrochloride (●), carbonate (▲), and borate (■), all at 50 mM.

p-NP or *o*-NP substrates (Table 2). A survey of phosphatase activities at pH levels from 11 to 4.5 revealed that the cell extract of *C. graniforme* contained phosphatase activity only in the alkaline range. Optimal activity for the alkaline phosphatase was at pH 9.5 (Fig. 2). The diminished rate of phosphatase activity with borate buffer may be attributed to metal ion chelation by borate, which suggests a role for metals in this alkaline phosphatase. While Cohen (9) found that Mg^{2+} stimulated the activity of alkaline phosphatase from *P. blakesleeanus*, we observed no change in alkaline phosphatase activity when 20 mM $MgCl_2$ was added to the reactions.

Approximately 50% of the alkaline phosphatase from *C. graniforme* was solubilized in the cell extract, and the remainder was associated with a cell wall fraction, as indicated by the presence of *N*-acetylglucosamine in the bound enzyme fraction. The soluble alkaline phosphatase from *C. graniforme* was subjected to various steps of fractionation, which resulted in an enzyme with a final purification 265 times that of the original enzyme (Table 3). The enzyme obtained from the Sephadex G-150 column was partially purified in that multiple proteins were obtained by acrylamide electrophoresis.

The optimum temperature for the purified alkaline phosphatase was 30°C, while the enzyme attached to the cell wall had maximum activity at 40°C (Fig. 3). Further evaluation of the effects of temperature on alkaline phosphatase activity was through Arrhenius plots (Fig. 4). A break in the graphic expression of the purified enzyme occurred at 15°C, with diminished activity above this temperature. The expression was linear across the incubation temperature, with the enzyme associated with the cell structures. The activation energy for the catalysis was calculated to be 9.9 kcal (1 cal = 4.184 J) for the alkaline phosphatase from *C. graniforme*.

The molecular weight of the purified enzyme was 60,000. Evaluation of enzyme kinetics revealed an apparent K_m of 0.21 μM for alkaline phosphatase at pH 9.5, with a V_{max} of 1 μmol of *p*-NP-phosphate hydrolyzed per min per mg of protein.

Evaluation of alkaline phosphatase levels in *C. graniforme* cultivated in different concentrations of P_i (Table 4) indicated that the production of enzyme was unchanged over a range of 3.7 to 31.5 mM phosphate. These results suggest

TABLE 2. Activity of hydrolytic enzymes present in cell extracts of *C. graniforme*

Substrate	U of enzyme activity (10^4) at pH of[a]:		
	4.5	7.0	9.5
p-NP-acetate	8.0	5.4	2.2
p-NP-α-D-galactopyranoside	5.7	5.1	NT
p-NP-β-D-galactopyranoside	5.9	5.5	4.8
p-NP-α-D-glucopyranoside	6.9	5.4	4.9
p-NP-β-D-glucopyranoside	27.0	5.9	6.4
p-NP-α-D-mannopyranoside	4.8	16.0	5.7
p-NP-β-D-mannopyranoside	0	0	0
p-NP-phosphate	19.0	22.0	29.0
p-NP-sulfate	0	0	0
p-NP-β-D-xylopyranoside	0	0.9	0.5

[a] 1 Unit equals 1 μmol of nitrophenol released per 90 min per mg of protein. NT, Not tested.

TABLE 3. Purification of alkaline phosphatase from *C. graniforme*

Procedure	Total protein (mg)	Total enzyme (U)	U/mg of protein	Yield (%)	Purification (fold)
Dialyzed cell extract	54.45	0.445	0.008	100	
80% Ammonium sulfate pellet	2.028	0.270	0.130	50	15
DEAE-cellulose	0.125	0.150	1.250	28	156
Sephadex G-150	0.080	0.107	2.120	3	265

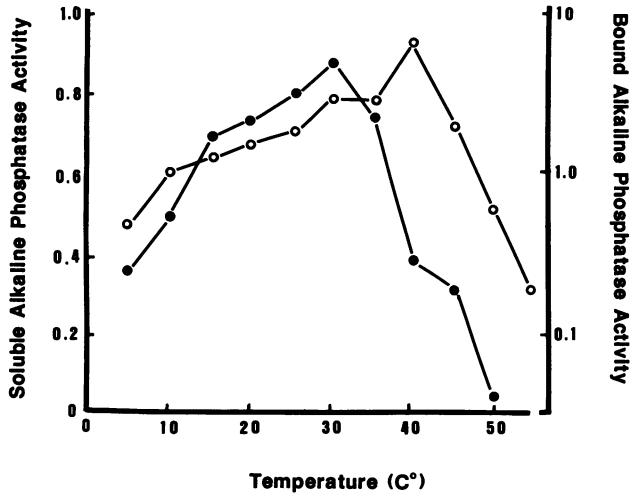

FIG. 3. Optimal temperature for alkaline phosphatase from *C. graniforme*. Activities are given in micromoles per minute per milligram of protein with bound enzyme (○) or soluble, purified enzyme (●).

that alkaline phosphatase in this fungus is constitutive under the conditions employed for growth, just as the acid phosphatase in certain mycorrhizal fungi (22) or rumen bacteria (14) was constitutive.

The survey of activity of culture filtrate from *C. graniforme* growing for 45 days on glucose indicated several enzymes (Table 5) that are capable of hydrolyzing NP compounds. Present were β-D-galactopyranosidase, β-D-glucopyranosidase, alkaline phosphatase, and β-D-xylopyranosidase. Absent from the culture filtrate were several activities reported in Table 2. No enzymes were found in the culture filtrate of *C. graniforme* grown for 30 days.

DISCUSSION

C. graniforme produced a variety of hydrolytic enzymes; however, rapidly growing cultures produced no cell-free exocellular enzymes. The enzymes present in culture filtrates of aged cultures reflect release of enzymes from cells and not true secretion. The presence of cell-bound hydrolases has been previously reported for bacteria (13, 16, 32) and ectomycorrhizal fungi (1, 4, 37) and would be significant in the fungus-root association. Disorganization of the mycorrhizae, with release of hydrolytic enzymes, occurs some distance from the area of new roots (19, 39) and would have little effect on the plant tissue or root physiology.

This demonstration of alkaline phosphatase in *C. graniforme* is the first report of an alkaline phosphatase in ectomycorrhizal fungi and provides a mechanism whereby phosphate can be utilized from organic phosphates in alkaline soils. It is curious that alkaline phosphatases have not been previously demonstrated in mycorrhizal fungi. Differing cultural and assay conditions could account for the specific phosphatases observed. However, since alkaline phosphatases contain metal cations (9, 23), buffers used in the assays would have to be free of metal chelators. Unfortunately, the buffers used for detection of mycorrhizal acid phosphatases (1, 2, 4, 22, 37) contained boric, citric, and maleic acids, which are excellent chelators of metal cations.

The energy of activation of this alkaline phosphatase is consistent with that given by other reports, in which the

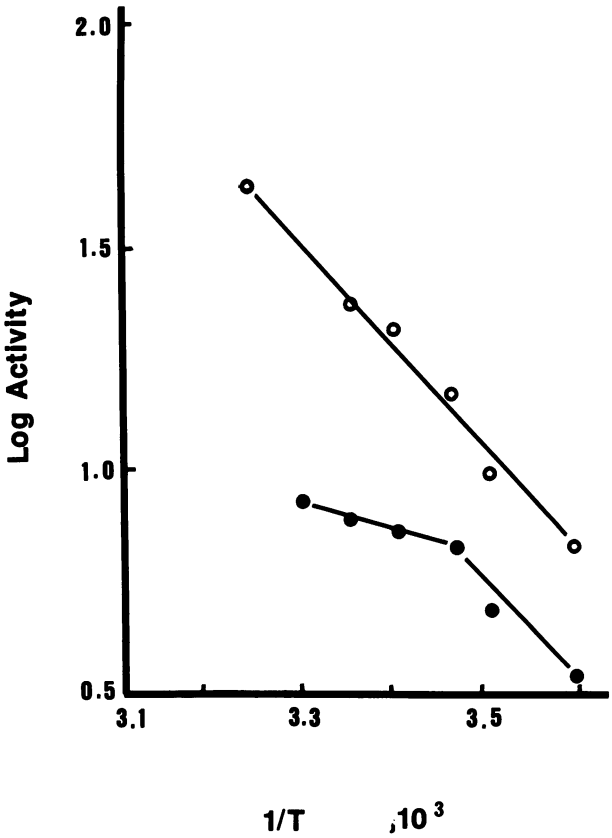

FIG. 4. Arrhenius plots of purified and bound alkaline phosphatase. Activation energies for the bound enzyme (○; 10^5) were 2.4 kcal/mol below 15°C and 9.9 kcal/mol above 15°C. For the purified enzyme (●), the activation energies were 9.9 kcal/mol below 15°C and 2.4 kcal/mol above 15°C. Alkaline phosphatase activity used for log activity was measured in micromoles per minute per milligram of protein.

catalysis ranged from 9.3 to 9.9 kcal (2, 9). Antibus et al. (2) found that surface acid phosphatases of several ectomycorrhizae produced linear Arrhenius plots over a broad temperature range, just as we reported here for the bound alkaline phosphatase. The break seen in the Arrhenius plot with the purified enzyme (Fig. 4) is consistent with results for the purified enzymes from *P. blakesleeanus* (9). This break in the Arrhenius plot indicates a conformational change in the purified enzyme, which would be in a nonphysiological condition. The cell wall material of *C. graniforme* influences the activation energy for alkaline phosphatase, just as the

TABLE 4. Alkaline phosphatase production by *C. graniforme* in media with different phosphate concentrations

Phosphate added to growth medium (mM)	Total protein (mg)/300 ml of culture	U[a] of alkaline phosphatase	
		Total in cells from 300-ml culture	Per mg of protein (10^3)
3.7	124.8	0.253	2.0
5.1	112.1	0.265	2.4
22.2	102.0	0.225	2.2
31.5	113.6	0.224	2.0

[a] 1 U equals 1 μmol of *p*-nitrophenol released per ml per mg of protein.

TABLE 5. Enzymes in *C. graniforme* culture fluid after 45 days of growth

Substrate	U of enzyme/90 min per mg of protein
p-NP-acetate	0
p-NP-α-D-galactopyranoside	0
o-NP-β-D-galactopyranoside	2.1×10^{-4}
p-NP-α-D-glucopyranoside	0
p-NP-β-D-glucopyranoside	3.2×10^{-4}
p-NP-α-D-mannopyranoside	0
p-NP-β-D-mannopyranoside	0
p-NP-phosphate	2.5×10^{-3}
p-NP-sulfate	0
p-NP-β-D-xylopyranoside	6.3×10^{-4}

cell wall lipopolysaccharide from *Pseudomonas aeruginosa* affects the alkaline phosphatase from that bacterium (13).

The lack of secreted hydrolytic enzymes in the culture medium of growing cultures of *C. graniforme* reflects something about the ecology of these organisms. The localization of hydrolases on the cell surface has been shown in other microbial systems (9, 13, 16, 32) and would target the product of hydrolysis for fungal uptake and not for use by microbes free in the rhizosphere. Indeed, the localization of the carbohydrases attacking the NP substrates is not known, and the carbohydrases may not be at the surface but may be in special vacuoles, as has been described elsewhere for other fungal enzymes (21, 26). The appearance of hydrolytic enzymes in culture filtrates of autolyzing fungal cells complicates the evaluation of exocellular production by mycorrhizae. Just as past experiments have been devoted to the detection of surface phosphatases (1, 4, 37), future experiments will address the issue of hydrolytic enzymes bound to the surface of the mycelium. Additionally, in growth experiments with complex carbohydrates, enzymes released by autolysis must also be considered as a factor contributing to the nutrition of ectomycorrhizal fungi.

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