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Starch metabolism in *Leucoagaricus gongylophorus*, the symbiotic fungus of leaf-cutting ants

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Summary

Leucoagaricus gongylophorus, the symbiotic fungus of the leaf-cutting ants, degrades starch, this degradation being supposed to occur in the plant material which leafcutters forage to the nests, generating most of the glucose which the ants utilize for food. In the present investigation, we show that laboratory cultures of *L. gongylophorus* produce extracellular α-amylase and maltase which degrade starch to glucose, reinforcing that the ants can obtain glucose from starch through the symbiotic fungus. Glucose was found to repress α-amylase and, more severely, maltase activity, thus repressing starch degradation by *L. gongylophorus*, so that we hypothesize that: (1) glucose down-regulation of starch degradation also occurs in the *Atta sexdens* fungus garden; (2) glucose consumption from the fungus garden by *A. sexdens* stimulates degradation of starch from plant material by *L. gongylophorus*, which may represent a mechanism by which leafcutters can control enzyme production by the symbiotic fungus. Since glucose is found in the fungus garden inside the nests, down-regulation of starch degradation by glucose is supposed to occur in the nest and play a part in the control of fungal enzyme production by leafcutters.

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Introduction

The fungus *Leucoagaricus gongylophorus* is the symbiotic partner of several species of leaf-cutting ants (Silva-Pinhati et al., 2004), living on cut leaves inside the nests, to form the so-called fungus garden (Weber, 1955). There, the fungus produces enzymes that degrade foliar polysaccharides into assimilable nutrients for the ants. Among these nutrients, glucose produced from plant material in the fungus garden seems to be the main food source supporting the ants (Silva et al., 2003). Glucose could also be shown to support *L. gongylophorus* growth in laboratory cultures (Siqueira et al., 1998).

Martin and Weber (1969) have proposed cellulase, which is produced by the symbiotic fungus, as the main operating enzyme to generate nutrients for the ants from cellulose degradation. This theory has been accepted for many years and was reinforced by findings that show *L. gongylophorus* to be able to degrade cellulose (Bacci et al., 1995). However, additional studies have indicated that cellulase is poorly produced by the symbiotic fungus compared to other polysaccharidases, such as pectinases, amylases and xylanases (Siqueira et al., 1998). Therefore, we have proposed starch degradation by *L. gongylophorus* as the main source of glucose in the fungus garden (Silva et al., 2003).

In the present investigation, we show new insights in starch metabolism by *L. gongylophorus*, by characterizing growth rate and α-amylase, maltase and glucose production by this fungus cultured on different carbon sources. Our results confirmed the production of extracellular glucose from starch by *L. gongylophorus* and showed that glucose represses the production of fungal α-amylase and maltase and, ultimately, starch degradation by the fungus. We propose that such repression is a mechanism by which leafcutters control enzyme production by the symbiotic fungus inside the nests.

Material and methods

Fungal cultivation

The strain B1-97 of *L. gongylophorus* Möller (Singer), isolated from *Atta sexdens* Linnaeus nest, was cultured at 25 °C in slanted medium A (Pagnocca et al., 1990) for 30 days, after which two loopful fungal mycelia were transferred to 250 mL flasks containing 50 mL YNB-glucose culture medium (75 mM citrate-phosphate buffer pH 5.0, 0.67 g 100 mL^{-1} Yeast Nitrogen Base (Difco, United States, catalog no. 100690) and 0.50 g 100 mL^{-1} glucose) and the flasks were statically incubated at 25 °C for 30 days. Mycelial mass was then harvested with a loop and suspended in 150 mL sterile water and mixed with a Potter device. Then, 1.0 mL of cell suspension (0.8 ± 0.02 mg cell mass, dry weight) was transferred to 125 mL flasks with 50 mL YNB medium containing one (0.5 g100 mL^{-1}) or two (0.25 g100 mL^{-1} each) of the following carbohydrates: (a) glucose (J.T. Baker, Mexico, catalog no. 1916-01); (b) starch (Sigma, Japan, catalog no. S 9765); (c) maltose (Sigma, Germany, catalog no. M 9171); (d) starch and maltose; (e) starch and glucose; (f) maltose and glucose. Twenty-four flasks of each culture medium were statically incubated at 25 °C, six of each were collected at each of the incubation times (10, 15, 20 and 30 days). Culture media (50 mL) were then filtered through a 0.45 μm filter, and 0.1 mL samples were used for glucose assay (see below). The remaining filtrate was dialyzed against distilled water (24 h, 6–9 °C, replacing the water after 12 h), concentrated by liophylization, and dissolved in 1.0 mL 75 mM citrate-phosphate buffer (pH 5.0), in order to form what we called the crude sample, which was glucose-free and utilized for enzymatic assays. The mycelium, which was collected from each of the 50 mL culture media, was used to determine cell mass, which was expressed in milligrams of dry weight. The experiment described here was carried out in duplicate.

Enzyme assay

α-amylase was assayed with 1.0 mL of 2.0 g 100 mL^{-1} starch solution in 75 mM citrate-phosphate buffer (pH 5.0) containing 2 mM calcium chloride. A similar procedure was used for maltase assay, with 2.0 g 100 mL^{-1} maltose as the substrate. Starch or maltose solutions were mixed with 1 mL 1/10 (v/v) crude sample and the reaction mixture was kept at 30 °C for 60 min. At zero, 15, 30, 45 and 60 min, two 0.1 mL samples were collected from the reaction mixture, boiled for 3 min and submitted to glucose analysis (maltase determination) or starch analysis (amylase determination). Glucose was assayed with 0.1 mL sample, 0.9 mL water and 1.0 mL of glucose oxydase reagent (Labtest, Brazil, catalog no. 34-E). After 15 min at 37 °C, the reaction mixture had its optical density (OD) at 505 nm determined and glucose concentration in the culture media was calculated by comparison with a glucose standard curve. Starch was assayed with 0.1 mL sample and 1.0 mL iodine reagent, this

reagent was prepared with 0.25 g 100 mL^{-1} iodine (Merck, Germany, catalog no. 4761) and 0.16 g 100 mL^{-1} potassium iodide (Mallinckrodt, France, catalog no. 1127), according to Bernfeld (1955). Starch concentration was determined by OD at 620 nm and a starch standard curve. Glucose or starch concentrations were plotted against the incubation time and the resulting curve slope was used for calculating enzyme activity. One amylase unit (U_A) corresponded to a decrease of 1 μg starch min^{-1} mL^{-1} of culture medium. One maltase unit (U_M) corresponded to the production of 1 μMol glucose min^{-1} mL^{-1} of culture medium.

Statistics

In order to detect differences in amylase or maltase production in distinct carbon sources, enzyme activity values obtained in the five culture media ($n = 48$ values for each culture medium for each enzyme) were compared to each other through the Tukey test (95% significance level) (Zar, 1996), which was also used for comparing cell mass production ($n = 48$ per culture medium).

Results

The fungus *L. gongylophorus* was cultured for 30 days at 25 °C in YNB broth containing glucose, starch, maltose, starch and maltose, starch and glucose, or maltose and glucose. The mycelial mass (mg of dry weight) produced was 22 ± 1.6, 15 ± 1.2, 15 ± 1.3, 16 ± 0.5, 19 ± 2.0 and 19 ± 0.6, respectively, values which are not significantly distinct from each other.

The fungus growing on media containing starch, maltose, or starch and maltose produced greater amounts of α-amylase (Fig. 1b–d) than those grown on culture media containing glucose, starch and glucose or maltose and glucose, indicating that glucose repressed α-amylase activity slightly

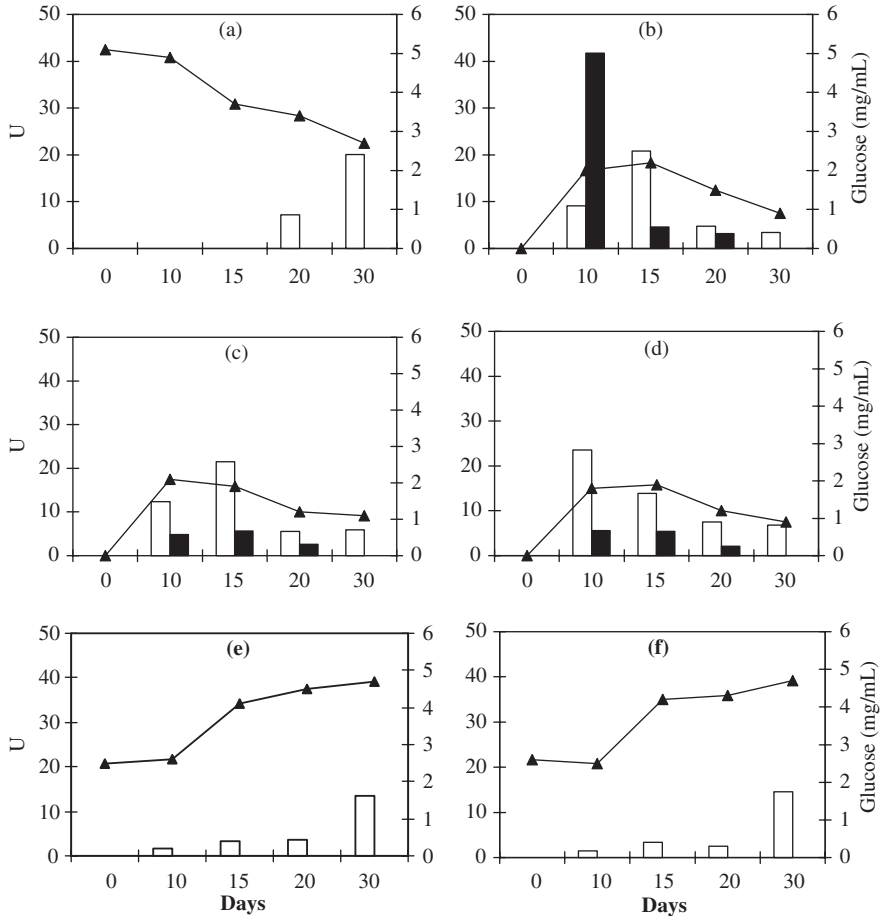

Figure 1. Units (U) of α-amylase (□) or maltase (■) and concentration (mg/mL) of glucose (▲) during cultivation of *Leucoagaricus gongylophorus* in different carbon sources: (a) glucose, (b) starch, (c) maltose, (d) starch and maltose, (e) starch and glucose and (f) maltose and glucose. Values of enzyme activity or glucose concentration for each of the incubation times corresponded to the means obtained from 12 replicates.

Table 1. α-amylase (U_A) or maltase (U_M) units (mean ± SD, $n = 48$) produced by *Leucoagaricus gongylophorus* on different carbon sources over entire period of growth

Carbon source	U_A	U_M
Glucose	7.5 (4.9)[a,b*]	0.0[c]
Starch	9.6 (4.2)[a]	12.7 (9.8)[a]
Maltose	11.2 (3.9)[a]	3.6 (1.3)[b]
Starch and maltose	13.1 (3.4)[a]	3.4 (1.5)[b]
Starch and glucose	5.0 (2.6)[b]	0.0[c]
Maltose and glucose	5.7 (3.7)[b]	0.0[c]

*Values followed by distinct letters are significantly different from each other (Tukey test).

(Fig. 1a, e and f). Similarly, starch was the best carbon source supporting maltase production, followed by maltose and starch plus maltose (Fig. 1b–d), as no maltase activity could be detected in media containing glucose, starch plus glucose nor maltose plus glucose (Fig. 1a, e and f). These results indicate that glucose severely represses maltase production by *L. gongylophorus*.

The higher sensitivity of maltase to glucose was characterized during growth on YNB containing glucose (Fig. 1a), during which no maltase was detected, whereas α-amylase production was very low during the first 10 days of incubation, and increased after 20 days, when glucose concentration decreased to $3.4 \pm 0.4\,mg\,mL^{-1}$. When *L. gongylophorus* was cultured on starch, glucose was produced in the culture medium, resulting in a dramatic decrease of maltase activity from the 10th to the 20th incubation day, as α-amylase activity increased in this period of time (Fig. 1b).

The total production of α-amylase over the entire period of growth indicated that starch or maltose, sole or mixed, are the best carbon source to induce α-amylase production by *L. gongylophorus*. For maltase production, starch was the best substrate (Table 1).

Discussion

In a previous work (Silva et al., 2003) it had been shown that glucose is the main nutrient supporting the survival of workers of *A. sexdens*, these workers are able to consume glucose which is generated apparently from plant material in the fungus garden. In the present investigation we found that *L. gongylophorus*, the fungus symbiotic with leaf-cutting ants, is able to grow on starch, maltose, glucose, or combinations of these carbon sources. During growth on starch or maltose, glucose was generated in the culture media, suggesting that glucose may be produced by *L. gongylophorus* through the degradation of the starch occurring in the plant material which is introduced in the nest by forager ants. These findings suggest that glucose production from plant material, through starch hydrolysis by fungal extracellular α-amylase and maltase, is a continuous process inside the ants' nest by which *L. gongylophorus* contributes to the ants' nutrition on starch. This process may be especially important for the ants, which apparently are not able to feed directly on starch, but readily utilize glucose (Silva et al., 2003).

The results also point to regulatory characteristics of enzyme production by *L. gongylophorus*, which produced α-amylase in all the carbon sources analyzed in the present investigation, indicating that α-amylase is constitutively produced by the fungus. However, this enzyme activity decreased when glucose was one of the carbon sources of the culture medium or when glucose was generated in the culture medium by the hydrolysis of maltose or starch, indicating that amylase can be somewhat repressed by glucose. A similar reduction in enzyme activity upon glucose production was also observed with maltase, but, contrarily to α-amylase, maltase was not produced when glucose was added to culture media, which indicates that maltase is more severely repressed by glucose.

Regulation of enzyme production by *L. gongylophorus* is similar to that seen in some *Aspergillus* species, in which the production of α-amylase on starch or maltose was higher than that on glucose (Lachmund et al., 1993; Nahas and Waldemarin, 2002). Also, regulation of enzyme production in *L. gongylophorus* resembles that seen in *Sulfolobus solfataricus*, in which glucose inhibits the production of α-amylase (Haseltine et al., 1996), as well as that seen in some species of *Termitomyces*, the fungi cultured by termites, in which starch induces the production of α-amylase and maltase (Mora and Rouland, 1995).

Glucose down-regulation of its own production from starch by *L. gongylophorus* is also expected to occur in the *A. sexdens* fungus garden, which was found to contain glucose (Silva et al., 2003). In addition, glucose was found to be consumed from the fungus garden by worker ants (Silva et al., 2003), so that this consumption is expected to stimulate α-amylase and maltase production by *L. gongylophorus* and, ultimately, starch degradation of plant material. Therefore, it is possible that leafcutters can control the amount of enzyme produced by their symbiotic fungus through glucose consumption from the fungus garden.

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