Developmental Regulation of Amylase Activity During Fruiting of *Schizophyllum commune*

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During the development of fruit bodies of *Schizophyllum commune* there is a minimum 10- to 15-fold increase in amylase activity. There is little or no activity in homokaryons or dikaryons. The activity is found early after the onset of morphogenesis and increases until the fruit bodies are mature. Inhibition studies with CO₂ indicate that the activity is directly associated with fruiting, as a change from fruiting to vegetative growth of the dikaryotic mycelium leads to a loss in activity, whereas the already formed fruit bodies show no loss. The activity is unaffected by the level of glucose in the medium. Evidence is presented, based on the mode of starch degradation and on yield and inhibition studies, that the enzyme is a glucosamylase.

*Schizophyllum commune*, a basidiomycete with a tetrapolar life cycle, has been used in a number of studies on differentiation and development. It possesses a relatively simple life cycle with several distinct developmental events and a usable genetic system (11). Homokaryotic vegetative mycelia of the proper mating type fuse to form a binucleate dikaryotic mycelium. Under the appropriate environmental conditions, fruiting bodies (basidiocarps) are formed from the dikaryon. These yield haploid meiotic products (basidiospores) which germinate to complete the life cycle.

This investigation concerns a change in enzyme activity associated with the formation of the fruiting body. Wessels (13) observed an increase in the level of an enzyme ("R-glucanase") which attacks part of the *S. commune* cell wall. The production of this enzyme is inhibited by glucose. The enzyme appears to be a β-1,6-glucanohydrolase (14).

To study the developmental genetics of fruiting in *S. commune*, additional changes in enzyme activity associated with fruiting must be identified. Preferably the enzymes studied should offer a reasonable opportunity for selection of mutants involved in their synthesis. During a study of the glycogen content of fruit bodies, a difference in the iodine-staining properties of the fruit bodies was observed. This led to an examination of the amylase activity in *S. commune*.

MATERIALS AND METHODS

Growth of cultures. Ten homokaryotic and seven dikaryotic strains of *S. commune* were examined. Cultures were grown by using fruiting minimal medium (glucose, 20 g; asparagine, 2.0 g; KH₂PO₄, 1.0 g; KH₂PO₄, 0.46 g; thiamine HCl, 120 μg; deionized water, 1 liter) on agar (2%) in standard petri dishes. Before inoculation, a sterile, water-permeable cellophane membrane was placed on the agar. Agar blocks from a previous culture served as inocula. Matings were made to fruit synchronously by placing them over watch glasses which contained 5% KOH and a solid CO₂ absorbent (Mallassorob, Mallinckrodt Chemical Works). By utilizing a pair of strains, one of which is a thin mutant (12), which show good fruiting competence, excellent synchrony is observed. By this technique, no difficulty is found in obtaining material at various stages of fruiting which is substantially free of nonfruiting mycelium (M. Schwalb, submitted for publication). Dikaryotic mycelia were prevented from fruiting by placing them under 5% CO₂ (9). All cultures were grown at 24 C. Cultures were harvested after 5 to 14 days of growth.

Preparation of extracts. Material was harvested by scraping the mycelium or fruiting material from the membranes and placing it in cold 0.1 M phosphate buffer, pH 6.2. The preparation was placed in an ice bath and macerated at 30,000 rev/min for 4 to 6 min (Virtis model 60K homogenizer). The macerated material was centrifuged at 8,000 × g for 10 min at 2 C and the supernatant fraction was used for subsequent assays.

Enzyme assays. Amylase activity was determined by placing a suitable quantity of extract (0.02 to 0.10 ml) in 1 ml of 0.15% glycogen (Shellfish, type II, Sigma Chemical Co.) in 0.1 M phosphate buffer, pH 6.0 (except as noted). The reaction was run at 37 C for 15 to 60 min, depending on the activity of the extract. Controls contained no substrate. Activity was determined by the 3,5-dinitrosalicylic acid (DNS) method (8). Absorbancy was read at 550 nm in a Gilford spectrophotometer. A standard curve was prepared by using...
D-glucose because of the high maltase activity in all extracts. Specific activity at 37°C was expressed as micromolar equivalents of glucose formed per minute per milligram of protein. Protein was determined by the method of Lowry et al. (7) with bovine serum albumin as a standard.

Glycogen phosphorylase was assayed spectrophotometrically. The reaction mixture contained phosphate buffer (pH 6.0), 100 μmoles; 0.15% glycogen; phosphoglucomutase, 1.3 units; glucose-6-phosphate dehydrogenase, 0.02 units; MgCl₂, 0.025 μmoles; nicotinamide adenine dinucleotide, 0.10 μmoles; and extract, 50 μlitters. The mixture was incubated at 37°C, and changes in optical density at 340 nm were measured in a Gilford recording spectrophotometer. Controls without glycogen were subtracted.

Glucose-1-phosphate phosphatase was determined by incubating 1 ml of 0.15% glucose-1-phosphate in 0.1 M phosphate buffer (pH 6.0) and 0.1 ml of extract at 37°C for 1 hr. Free glucose was determined by the glucose oxidase method (Glucostat Special, Worthington Biochemical Corp.).

Dextrinogenic and saccharogenic assays. To determine the loss of blue value of starch ("dextrinogenic activity") with enzyme extracts, a modification of the procedure of Hsiu et al. (2) was used. To a reaction solution containing 3.5 ml of 0.2% starch in 0.1 M succinate-NaOH buffer (pH 6.0) was added 1 ml of appropriately diluted enzyme solution. The mixture was incubated at 25°C. At various times, a 0.5-ml sample was removed and assayed for reducing groups by the DNS method, and another 1.0-ml sample was placed in a boiling water bath for 10 min and then assayed for glucose by the glucostat method. One milliliter of 1 N HCl was added to the remaining solution, followed by 40 ml of water and 0.5 ml of iodine solution (3.0 g of KI and 0.130 g of iodine in 100 ml of water). The absorption was read in a Klett-Summerson colorimeter with a no. 66 filter. The control contained no enzyme.

RESULTS

Characterization of the enzyme. Activity in crude extracts from mature fruit bodies was found against glycogen, starch, amylase, maltose, and starch. The activity toward glycogen, determined by using a variety of buffers, was found over a broad range of pH (about 2.2 to 8.5) with a peak at 5.6 to 6.0 in succinate-NaOH buffer. The activity in phosphate buffer was about 20% less than that in succinate buffer at pH 6.0.

To determine whether the enzyme was an endoenzyme (α-amylase) or an exoenzyme (glucoamylase), a comparison was made of the "dextrinogenic" (iodine-staining reaction) and "saccharogenic" (production of reducing groups) activity. The results (Fig. 1) indicate that the loss of "blue value" per unit production of reducing equivalents of glucose from S. commune enzyme proceeded at a slow rate. In addition, glucose was produced very early in the reaction, and the reducing equivalents of glucose almost equaled the amount of glucose produced when measured by the glucose oxidase method.

Table 1 lists the effects of various potential inhibitors or activators of the amylase activity. The only compounds to show significant inhibition were mercuric chloride, iodoacetate, and tris(hydroxymethyl)aminomethane (Tris). Ferric chloride and ethylenediaminetetraacetic acid also showed some inhibition of activity.

The extract was allowed to incubate for 18 hr at 37°C with 0.15% glycogen in 0.1 M phosphate buffer (pH 6.0) with or without a layer of toluene on top. The reaction mixture was assayed for glucose by the glucose oxidase assay. The molar yield of glucose ranged from 84 to 97% in various experiments.

Changes in activity during development. Table 2 summarizes the glycogen-degrading activity of extracts from normal homokaryons, dikaryons and fruit bodies. There is relatively low activity in homokaryons and dikaryons but a significant increase in fruit bodies. The low levels of activity found in some homokaryons and dikaryons may be attributable in part, or exclusively, to the presence of glycogen phosphorylase and glucose-1-phosphate phosphatase. Activity for both of these enzymes was found in homokaryons and dikaryons when examined at the same pH as in the amylase assay. However, although phosphatase activity was present, no glycogen phosphorylase was found in fruit body extracts under these conditions.

To test the possibility that the amylase activity might be environmentally controlled, two homokaryons were grown over KOH, and a fruiting culture was transferred daily to fresh medium. No increase in activity was found in the homokaryons, and the fruit bodies still produced about 90% of the control activity, well within the range of activities (Table 2) found under normal growth conditions.

Synchronously developing cultures were examined at four stages of development (Fig. 2), as described by Leonard and Dick (6). No Stage II structures were examined because of the difficulty in harvesting material from this transient period.

The results indicate that the enzyme was produced shortly after the initiation of development. At the earliest recognizable morphological event (Stage I) about 32% of the maximal activity was present. No loss of activity was observed in older fruit bodies. In order to determine whether aging plays a role in the increase in activity found in older fruit bodies, three dikaryons were placed in 5% CO₂ to prevent fruiting and were tested up to 14 days. The activity never reached more than 15% of the activity found in mature fruit bodies (Stage V).
To examine further the relationship of morphological development and commitment to amylase activity, a study of the effect of CO$_2$ inhibition was made (9). Cultures were made to fruit synchronously and at various stages were placed in 5% CO$_2$. This stopped further development of the fruit bodies except for those already entering Stage V. Lateral vegetative growth of the dikaryotic mycelium, which was inhibited in synchronous development, was allowed to continue. (Table 3). In the early stages there was some loss of activity after CO$_2$ inhibition. At Stage III or later no loss in activity was observed. In addition, the dikaryotic mycelia formed after the switch in morphogenesis had the highest activities ever found for the strains tested. They were, however, appreciably lower than the fruiting material which arose from the same dikaryon.
DISCUSSION

There are several lines of evidence to suggest that the amylase activity of S. commune is primarily due to a glucoamylase (α-1,4-glucan glucohydrolase, EC 3.2.1.3). First is the pattern of starch degradation. The enzyme produces relatively large amounts of reducing groups for each unit loss of iodine-staining reaction. In addition, glucose is produced early in the reaction. The amount of glucose produced when measured by the DNS method (reducing groups) or by the more specific glucose oxidase method is almost identical. This suggests that there is little, if any, internal chain breakage. The number of reducing groups is slightly higher than the amount of glucose produced, however, suggesting that some α-amylase is present. This situation is commonly found in the fungi (3, 10). Second is the inhibition of the amylase activity by heavy metals (mercuric and ferric ions) and by iodoacetate. This indicates an essential thiol group in the S. commune enzyme. It is generally accepted (1) that thiol groups are not found in α-amylase. In addition, the glucoamylase of another fungus, Coniphora cerebella, is inhibited by HgCl₂ and Tris (4, 5). Last, the high yields are suggestive of a glucoamylase (10). The presence of debranching enzymes is an alternate explanation for the almost total breakdown of glycogen observed.

The results demonstrate that the amylase activity is specifically related to fruiting. A minimum 10- to 15-fold increase in specific activity is observed during fruiting. The exact increase varies between strains and is also dependent on whether the low levels of activity found in some homokaryons and dikaryons really results from amylase.

The increase in amylase activity is not affected by environmental changes which do not affect fruiting itself (i.e., homokaryons over KOH, fruit bodies with a relatively constant supply of glucose, or aging). The lack of glucose inhibition of amylase activity in fruiting cultures demonstrates that the increase in activity is not related to starvation or nutritional balance. This is in contrast to previous reports of R-glucanase (13). The R-glucanase is definitely regulated by the level of glucose in the medium, starvation causing an increase in enzyme activity while a constant supply of glucose represses activity. The assumption that the R-glucanase is developmentally regulated was based on the observation that the constant supply of glucose inhibited fruiting as well. However, the medium also lacked a nitrogen source. In the present study, no inhibition of fruiting by glucose at the 2% level is seen when the complete medium is changed daily (M. Schwalb, submitted for publication). In comparing the results reported here for glucoamylase activity and those reported for R-glucanase, it would seem that the question of the relationship of the R-glucanase to fruiting is still open. Both R-glucanase and amylase activity were found associated with the B mating-type factor of S. commune (15). Control of the amylase activity in fruiting may occur by a different mechanism, as nonfruiting dikaryons, all of which are heterozygous at the B factor, show no activity.

The glucoamylase activity rises early in the development of fruit bodies and continues to increase until the end of the morphogenetic sequence. Whether this represents de novo synthesis through any or all of the sequence has not yet been determined.

The relationship between CO₂ and the initiation of fruiting has been documented (9). Vegetatively growing dikaryotic mycelia resulting from the CO₂ inhibition of fruiting have the amylase activity found in dikaryons which never fruit and not the level found in fruit bodies formed under CO₂-free conditions. That is, the dikaryotic mycelium becomes committed concurrently to fruiting and the appearance of amylase activity, and when it loses the morphogenetic commitment, amylase activity is also lost. In addition, preformed amylase activity is not lost in the presence of CO₂, as long as the level of morphogenesis remains unchanged. These results further demonstrate the relationship of amylase activity to fruiting morphogenesis.

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LITERATURE CITED


