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Changes in the Carbohydrates of *Allomyces macrogynus* during the Selective Development of either Zoosporangia or Resistant Sporangia

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Abstract

Allomyces macrogynus synthesized trehalose but did not appear to metabolize it. Trehalose was released to the suspending medium before the development of zoosporangia by plants suspended in water and before the development of resistant sporangia by plants in glutamic acid and glucose solution. Plants in the latter solution accumulated high levels of glycogen and trehalose before mRNA was transcribed for the development of resistant sporangia. Before the transcription of mRNA for either zoosporangia or resistant sporangia there was a period during which the plant content of glycogen, trehalose, protein and nucleic acid was constant. The increase in wall glycans and chitin during the development of zoosporangia was measured and possible origins of the walls are discussed.

Introduction

Allomyces macrogynus was induced to make zoosporangia, by suspension of vegetative plants in distilled water or resistant sporangia in glucose–glutamic acid solution (Youatt 1980*a*). Analysis of the supernatant media and the contents of the plants showed that plants which produced zoosporangia degraded RNA during this development, releasing phosphate, uracil, guanine and hypoxanthine. Plants which produced resistant sporangia also degraded RNA and released uracil and the purines but accumulated bound phosphate in the low molecular weight material of the cells (Youatt 1980*b*).

This paper concerns analyses of reserve carbohydrates and amino acids on the same supernatants and extracts as described above. The purpose here is to determine how the supplied nutrients, glucose and glutamic acid, control the differentiation process.

Plants which produce zoosporangia in distilled water must make all the new structures from existing structures or reserve materials. Morrison (1977), from electron microscopic studies, found little evidence of reserve materials and suggested that new structures were made from existing structures such as the endoplasmic reticulum. The analytical results are also considered in this context.

Methods

Detailed descriptions of the culture methods and microscopic observations have been reported (Youatt 1980*a*). The fractions analysed in this paper were identical with those described previously (Youatt 1980*b*). The samples for analysis were: the supernatant media [distilled water for the production of zoosporangia and glucose–glutamic acid for resistant sporangia], the contents extracted by ethanol and by acid, the contents extracted by 0·1 M KOH and walls dissolved in 10 M HCl.

Trehalose Assay

Supernatants from plants suspended in distilled water were concentrated in a rotary evaporator at 45°C. A low molecular weight fraction was separated by gel filtration using Biogel P2 and desalted by ion exchange. Trehalose was then separated by paper chromatography.

The chromatographic systems used for preparation and identification of trehalose were: paper chromatography on Whatman No. 1 paper with 80% (v/v) isopropanol in water or ethyl acetate–pyridine–water (12:5:4 v/v); thin-layer chromatography with silica gel impregnated with 0·2 M Na_2HPO_4 and developed with n-propanol–pyridine–water (5:3:2 v/v); gas–liquid chromatography on SE 30 of the trimethylsilyl derivative (Sweeley *et al.* 1966). Trehalose was detected after dipping in $AgNO_3$ in acetone then in KOH in ethanol (Wheat 1966).

Trehalose was hydrolysed quantitatively in 3 M HCl for 1 h at 100°C and the released glucose was assayed with a glucose oxidase–peroxidase kit according to Sigma Technical Bulletin No. 510. The stability of trehalose in alkali was demonstrated when samples were heated in 24% (w/v) KOH at 100°C for 20 min and assayed, using the anthrone reagent, as described below.

Assays of Glucans and Galactan

Glucose, glycogen, trehalose, wall glucan and wall galactan all reacted with anthrone. Bound glycogen and wall glycans had to be assayed in the presence of protein. Interference by protein was minimized by reducing the concentration of anthrone and acid in the reaction mixture. The procedure used was as follows: reagent, prepared daily, contained 50 mg anthrone in 50 ml conc. H_2SO_4. Samples (0·5 ml) were placed in 150 by 6-mm test tubes and 0·75 ml of reagent was carefully layered underneath. All the samples were then mixed thoroughly and transferred to a boiling water bath for 10 min. Standard solutions were prepared with the same acid or alkaline content as the fractions being analysed.

Supernatants from plants producing zoosporangia contained trehalose as the only detectable sugar and the anthrone reagent was used for assay. Supernatants from plants suspended in the glucose–glutamic acid series required the analysis of glucose and trehalose in the presence of each other. Glucose was assayed by the Sigma glucose oxidase–peroxidase method. Trehalose was assayed after alkaline destruction of glucose, using the anthrone procedure. The standard glucose assay kit gave positive reactions with some α-linked glucosides, including glycogen, but did not give a reaction with trehalose.

Soluble glycogen and trehalose, both alkali-stable, were present in the ethanol–acid extracts. Two procedures were used. Glycogen was precipitated by ethanol in the presence of sodium sulfate (Rickenberg *et al.* 1975) and assayed with anthrone. Trehalose was assayed on the dried ethanol supernatants and correction applied for the saturation concentration of glycogen. When an Amicon ultrafiltration system became available the preferred method was to assay glycogen and trehalose together and then trehalose in the ultrafiltrate, obtaining glycogen by difference.

Chitin Assay

Complete hydrolysis of chitin in the wall extracts was achieved in 6 M HCl at 100°C for 3 h. Acid was removed in a stream of air without loss of glucosamine. Separation of glucosamine on a cation exchanger proved to be unnecessary and, after a comparison of available methods for the assay of glucosamine, Johnson's (1971) assay was used.

Assay of Amino Acids

Qualitative analysis of supernatants and ethanol–acid extracts was by paper chromatography on Whatman No. 1 paper using butanol–acetic acid–water (4:1:1 v/v) in one dimension and 80% (w/v) phenol containing 1% (w/w) ammonia in the other. Semiquantitative comparisons were made by comparison of the intensity of the colour developed with ninhydrin. In a test tube 0·2 ml of supernatant medium was heated to dryness with 1 ml 0·2% (w/v) ninhydrin in ethanol, in a boiling water bath. The residue was dissolved in ethanol and the colour measured at 570 nm compared with standard leucine in the range 20–100 μg/ml.

Reagents

Acetylacetone was obtained from Hopkins and Williams (U.K.) and distilled before use. All biochemical reagents were obtained from the Sigma Chemical Co. (U.S.A.).

Results

Trehalose

Trehalose has not previously been described in *Allomyces* spp. It was isolated as described and identified as a non-reducing sugar which was stable to akali and hydrolysed to glucose. It chromatographed with authentic α,α-trehalose, or its trimethylsilyl derivative, in the systems described in the Methods.

Trehalose was supplied to *A. macrogynus* growing in a medium which contained amino acids as the only alternative source of carbon. Neither the rate nor the growth yield was increased and, 4 days after all growth ceased, there was no detectable loss of trehalose. Suspensions of plants which completely metabolized additions of glucose and maltose in 5 min did not reduce the concentration of trehalose in 6 h.

Amino Acids

The main amino acids detected in the supernatant media and those found in the ethanol–acid extracts were the same. They were alanine, glycine, serine, glutamic and aspartic acids.

Glycogen

Soluble glycogen was found in ethanol–acid extracts and bound glycogen in 0·1 M KOH extracts. The stability of both to alkali was established, as for trehalose. The wall extracts contained no alkali-resistant glucan, confirming that all the glycogen had been extracted. Wall extracts contain glucan, galactan and chitin as the main carbohydrates (Youatt 1977).

Changes in the Supernatant Medium

When vegetative plants were transferred from growth medium to water or various buffer solutions at room temperature or 30°C there was a rapid loss of trehalose and amino acids to the supernatant medium (Youatt, unpublished data). Further transfers resulted in further losses. These observations suggested equilibration processes of some complexity. This phase of response was complete in 10–20 min and is not included in the present series of samples.

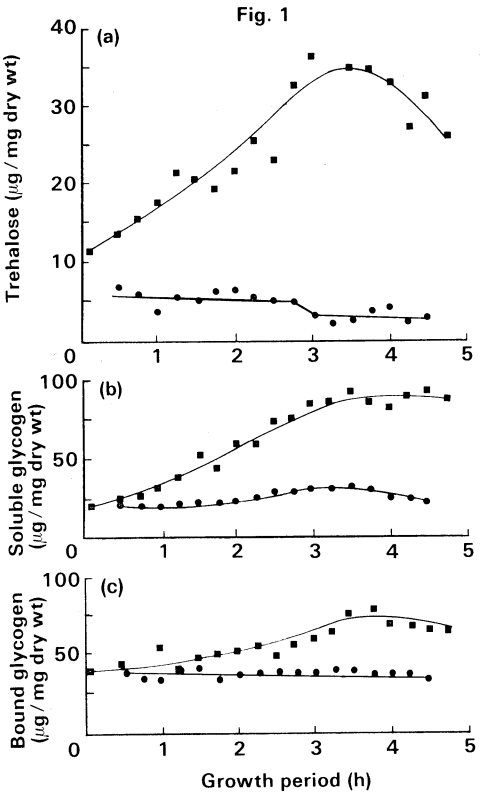
From 30 min until spore release at 165–180 min supernatants of suspensions of plants in distilled water had constant low levels of amino acids and 10 μg/ml of trehalose. No other sugars were present in assayable amounts. After spore release there was a small release of glycopeptide from the discharge plug and associated loss of glycogen and probably also of trehalose (Youatt 1976). The amounts released were too small for more detailed analysis. Amino acids and peptides also arose from the digestion of the discharge plug.

Plants suspended in glucose–glutamic acid solution made only occasional zoosporangia in the first 3 h. They underwent hyphal elongation from 150 to 200 min and began to produce resistant sporangia at 300 min. In the presence of a large excess of glucose the low initial level of trehalose was not accurately assessable but the concentration rose from 10–20 to 40–50 μg/ml between 180 and 285 min. This final level represented approximately 48 μg trehalose per milligram dry weight of plants.

The high initial concentration of glutamic acid made the initial detection of leaked amino acids difficult but at 285 min, when glutamic acid had largely been metabolized,

the amino acid composition of the supernatant was similar to that of the distilled water series.

Glucose in the supernatant was reduced from 860 to 350 μg/ml in the first 240 min and to 220 μg/ml at 270 min. There was, therefore, glucose available to the plants at all times. Glutamic acid content fell by 20% in the first 90 min. There was then a steep decline during the period of hyphal elongation with 80% loss by 240 min.



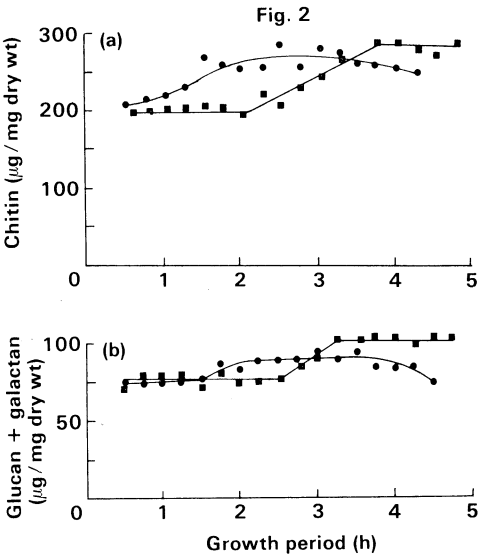


Fig. 1. Changes in trehalose and glycogen content of *A. macrogynus* during growth. Analyses of plants suspended in glucose–glutamic acid solution to make resistant sporangia (■) or in distilled water to make zoosporangia (•). (*a*) Trehalose in the ethanol–acid extract. (*b*) Soluble glycogen in the ethanol–acid extract. (*c*) Bound glycogen in the alkaline extract as described in the Methods. Results for plants in glucose–glutamic acid solution are related to the initial dry weight of the suspension.

Fig. 2. Analysis of polysaccharides in walls of *A. macrogynus* during growth. ■ Plants suspended in glucose–glutamic acid solution to make resistant sporangia. • Plants suspended in distilled water to make zoosporangia. (*a*) Chitin was hydrolysed and analysed as described in the Methods and related to the initial dry weight of plants. (*b*) Glucan and galactan assayed together as described and related to the initial dry weight.

Changes in the Carbohydrate Content of Plants

Fig. 1*a* compares the trehalose content of plants of the two series. Plants which made zoosporangia had a low content of trehalose with either a small decline in concentration or, possibly, a loss at the time of spore release (165–180 min). Plants supplied with glucose and glutamic acid synthesized trehalose for 3 h but ceased to

accumulate trehalose at a time when glucose was still present. The fall in the trehalose content from 4 h was associated with a rising concentration of trehalose in the supernatant medium and does not, therefore, represent a utilization of trehalose by the plants.

Figs 1*b* and 1*c* show changes in the glycogen content of plants for both series. Plants in distilled water had a steady low concentration of soluble glycogen until the zoosporangium appeared complete (120 min). The soluble glycogen content then rose until the time of spore release (180 min). Although these changes do not appear large they were of the order of a 50% increase and seen always at this time in many other experiments. The later decline after spore release has also been a common feature. Plants suspended in glucose–glutamic acid solution accumulated both soluble and bound glycogen until 3 h, as they accumulated trehalose.

Changes in the Walls of Plants

From the size of plants and their zoosporangia it was estimated that an increase in wall material of about 20% might be observed. An increase of this order was found in the chitin content (Fig. 2*a*) and the glycan content (Fig. 2*b*). The rise occurs during the development of the zoosporangia and is not seen in the analyses of plants from glucose and glutamic acid which only made an occasional zoosporangium. These plants show an increase in the wall components at the time of hyphal elongation (150–200 min). This experiment does not span the time of formation of the resistant sporangium. The small rise and later decline in wall components of plants suspended in water has been confirmed in other experiments.

Discussion

Vegetative plants of *A. macrogynus* transferred to solutions of glucose alone produced zoosporangia and released spores in 3 h; when transferred to glutamic acid alone they produced spores only after a long time lag (Youatt *et al.* 1971). Therefore it is glutamic acid which prevents the development of zoosporangia in the glucose–glutamic acid solution. It appears that the transcription of mRNA was inhibited, since a transcription at 4 h was necessary for the development of resistant sporangia (Youatt 1980*a*).

The role of glucose seems to be in part related to the accumulation of glycogen and trehalose. When the first transcription is not blocked, plants in glucose produce a succession of zoosporangia. However, the role of glucose is more complex than this. In glucose–glutamic acid solution, zoosporangia develop if the supply of glucose is exhausted before transcription.

It is clear from these considerations that times of particular interest are the periods 20–40 min and 200–240 min. During the first period, plants in water, which produced zoosporangia, had steady external concentrations of trehalose and amino acids and, from a previous study (Youatt 1980*b*), of phosphate. Internal concentrations of trehalose, glycogen, protein and nucleic acid were also stable. By contrast, plants in glucose–glutamic acid medium, which did not make zoosporangia, were accumulating glycogen and trehalose and degrading nucleic acid during the first period. During the second period, when these plants transcribed mRNA for the development of resistant sporangia, they also had steady internal concentrations of glycogen, trehalose and nucleic acid. These observations suggest a necessity for the plants to shut off

some of their metabolic activities when transcription of mRNA is to take place. This may be difficult when an excess of a single amino acid is present.

The role of trehalose in *A. macrogynus* is obscure at present. The organisms synthesize the compound but appear not to use it. A decline in the contents of the plants was always associated with a loss of trehalose to the supernatant. Experiments were carried out with [^{14}C]trehalose but were discontinued because much of the evidence suggested that there was a persistent contaminant in the radioactive preparation. Other approaches to the problem are being sought because it appears possible that if trehalose does not serve as a nutrient it may have a regulatory role. Somewhat similar observations have been made with yeasts. Organisms which were unable to use trehalose were nevertheless found to contain trehalase (Barnett 1976).

The question of the origin of newly synthesized wall material during the production of zoosporangia has also been considered using data from this and a previous paper (Youatt 1980*b*). The possible origins considered were glycogen, trehalose, ribose from degraded RNA, protein, amino acids or lipid. At the time of wall synthesis only the concentration of RNA was falling and, as no ribose accumulated, its metabolism was assumed to have occurred. The amount of ribose released, calculated from the change in phosphate, was insufficient to account for the wall synthesis but may have contributed to it. Plant contents were hydrolysed and chromatographed but no evidence of other reserve materials or precursors of chitin were found at the time preceding wall synthesis. The results therefore suggest *de novo* synthesis from other metabolic pools which were built up in the first 20 min, when the glycogen content fell, and which were replenished later from various possible sources such as ribose from RNA and the late decline in glycogen and wall substance.

The development of sporangia in *A. macrogynus* shows much promise as an experimental system for studies of differentiation. It is particularly convenient that stages of development are so readily monitored.

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