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Formation of *O*-Ethylhomoserine during the Controlled Production of Resistant Sporangia in *Allomyces macrogynus*

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Abstract

Evidence is presented that *O*-ethylhomoserine which accumulates in the amino acid pools of *A. macrogynus* before the development of resistant sporangia is synthesized from homoserine and ethanol, the enzyme being produced in response to methionine deficiency. A requirement for suboptimal aeration in the development of the sporangia is accounted for by a need to produce ethanol from glucose. *A. macrogynus* has a limited ability to synthesize methionine and it is suggested that accumulation of *O*-ethylhomoserine inhibits this synthesis.

Extra keywords: hyphal branching; cystathionine.

Introduction

In growing cultures of *Allomyces macrogynus* limited growth was observed without added methionine. Below concentrations of 60 μM L-methionine plants were unable to branch normally. At and below concentrations of 100 μM plants produced resistant sporangia when other essential requirements—reduced aeration and the presence of excess glucose—were met, the suboptimal aeration allowing for some fermentation of glucose to ethanol whilst still providing sufficient energy for the many synthetic activities required for the development of a sporangium. Optimum growth was achieved at concentrations of 250 μM L-methionine, and in the presence of excess methionine only zoosporangia were produced (Youatt 1982*a*). The requirement for methionine was thus linked to poor branching and the production of resistant sporangia. An association between morphological development and the balance of oxidative and fermentative metabolism has also been found in organisms which can exist in yeast or mycelial forms (Terenzi and Storck 1969).

In this paper the presence of *O*-ethylhomoserine in the amino acid pools of *A. macrogynus* plants about to produce resistant sporangia is demonstrated. A limited range of microorganisms has been shown to make this ether when supplied with ethanol and homoserine (Murooka and Harada 1967, 1968), the enzyme involved being the second enzyme of the biosynthetic pathway to methionine.

Methods

Culture methods have been described previously (Youatt 1980*a*) and the bulk medium used was described by Sandars and Youatt (1983). Extraction of amino acid pools, trehalose and glycogen were described by Youatt (1980*a*) and assay procedures for carbohydrates by Youatt (1980*b*).

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Paper Chromatography

O-Ethylhomoserine was separated from all amino acids tested except proline by 80% (w/v) phenol. This pair of amino acids was not usually present but could be separated in two dimensions with the further use of butanol–acetic acid–water (4 : 1 : 1 v/v). Threonine was qualitatively demonstrated with 80% (w/v) phenol but in isolating a sample for n.m.r. spectroscopy the solvent t-butanol–water–methyl ethyl ketone and diethylamine (10 : 10 : 5 : 1 v/v) was used to obtain threonine free of homoserine and alanine.

Automatic Amino Acid Analysis

O-Ethylhomoserine eluted in 123–125 min at 60°C using a Jeol Analyser JL C6AH with a column 63 by 0·8 cm and citrate buffers of pH 3·00, 3·25 and 4·25. The ether emerged between proline and glycine. Homoserine emerged with glutamic acid.

Mass Spectroscopy

The methyl ester of *O*-ethylhomoserine was produced by reaction with pre-mixed dry methanol and redistilled thionyl chloride. Dansylation was carried out subsequently (Gray 1967). The mass spectrum was obtained at 200°C and 70 eV after direct insertion of the sample.

Results

Identification of O-*Ethylhomoserine*

The amino acid was obtained after plants had been incubated for 6–8 h with glucose and amino acids favourable to the formation of resistant sporangia (Sandars and Youatt 1983). Approximately 2 mg of material was isolated by paper chromatography using 80% (w/v) phenol and subsequently butanol–acetic acid solvents. Part of the sample was converted to the dansyl methyl ester derivative. The accurate mass of the derivative gave $C_6H_{13}NO_3$ as the molecular formula of the original amino acid. An n.m.r. spectrum in D_2O showed a triplet at 1·18 and a complex absorption at 3·56 suggesting the presence of an amino acid with an ethyl ether group. A similar spectrum had been reported for *O*-ethylhomoserine (Murooka and Harada 1967). The n.m.r. spectrum in D_2O of pure synthetic L-*O*-ethylhomoserine showed minor differences from the spectrum of the ether obtained from *A. macrogynus* but these proved to be due to differences in pH of the solutions. The authentic sample eluted from the column in automatic amino acid analysis at the same time as the material from *A. macrogynus*. Automatic amino acid analysis is well suited for the detection of the ether since it is well separated from the nearest amino acids, proline and glycine.

Production of O-*Ethylhomoserine by* A. macrogynus

A. macrogynus suspensions in glucose (12 mM), homoserine (2 mM) and ethanol (170 mM) produced much more of the ether than had previously been observed. The ether accumulated in the amino acid pools and remained present at 24 h in agreement with the observations of Murooka *et al.* (1977) that the ether, once formed, is stable.

In the study of the role of amino acids (Sandars and Youatt 1983) the requirements for the production of resistant sporangia were defined. In all conditions which allowed the development of resistant sporangia *O*-ethylhomoserine was detected and, except at trace concentrations, was detected in no other conditions. The two amino acids, aspartic and glutamic acids, which are not accumulated at high concentration in the amino acid pools, were unable to prevent the initial production of zoosporangia but both amino acids were favourable to later production of resistant sporangia. The ether was found at that time with these two amino acids as well as with arginine, asparagine, cysteine, histidine, glycine, leucine or valine used in the conditions described previously (Sandars and Youatt 1983). The ether has not been found in plants which were suspended in glucose with alanine, isoleucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan or tyrosine tested over a range of concentrations.

Externally Supplied O-Ethylhomoserine

Plants suspended in 12 mM glucose and 2 mM *O*-ethylhomoserine had taken up detectable amounts of the ether into the amino acid pools in 5 min and retained high internal concentrations the following day, long after the ether was gone from the medium. Apart from the ether the amino acid pools retained only concentrations of alanine, glutamic and aspartic acids, glycine and serine such as were seen in starving plants (Youatt 1980*b*).

In the suspending medium containing glucose and *O*-ethylhomoserine *A. macrogynus* immediately underwent hyphal elongation with some branching. At 8–9 h resistant sporangia were produced in the terminal position. The shape of the developing sporangium was more elongated than with other amino acids but normal development of melanin and acid resistance followed. Trehalose accumulated to a higher concentration than in controls with glucose only—in one experiment to 98 μg trehalose per milligram dry weight compared with 50 μm/mg in the control. This was reminiscent of the effect of cycloheximide which also altered the maximum content of trehalose reached (Youatt 1982*b*).

Externally Supplied Homoserine

Plants suspended in 12 mM glucose and 2 mM homoserine had accumulated detectable homoserine in the amino acid pools in 5 min. Hyphal elongation was severely inhibited until the homoserine content of the medium had declined below detectable levels at 5–6 h. The plants then made zoosporangia.

The presence of homoserine allowed normal accumulation of glycogen but by 1 h the synthesis of trehalose was inhibited and trehalose was released from the plants. Trehalose synthesis recommenced at 5 h after homoserine had been metabolized. A similar inhibition of the synthesis was observed with valine and threonine at 4 mM but not at 1 mM concentration. Measured over a period of 3 h 2 mM homoserine reduced the rate at which 4·5 mM glucose was utilized by 40%. The effect of this inhibition of glucose metabolism was similar to the effect observed previously of an exhaustion of the supply of glucose (Youatt 1982*c*).

Plants were suspended in 12 mM glucose, 2 mM homoserine and 170 mM ethanol and samples were taken at intervals of 2 h. The amino acid pools were extracted and chromatographed with 80% (w/v) phenol. After 2 h there was considerable conversion to threonine. The identity of threonine was confirmed by automatic amino acid analysis and the n.m.r. spectrum of an isolated sample. *O*-Ethylhomoserine was first detected at 4 h and reached its peak concentration at 6–8 h as the homoserine became undetectable in the pools. The presence of ethanol increased by approximately 10-fold the amount of *O*-ethylhomoserine produced but did not allow it to be produced any earlier than 4 h. Since the defined medium used for the culture of the organisms contained no threonine the biosynthetic pathway aspartic acid–homoserine–threonine was, presumably, already present and plants were immediately able to convert supplied homoserine to threonine. The growth medium was supplied with methionine and hence, at the time of harvesting, the enzymes for the conversion of homoserine to methionine were absent. As the suspension was methionine-deficient it was concluded that the lag in the production of *O*-ethylhomoserine was due to the time taken to make the necessary enzymes.

To test the validity of this conclusion plants were suspended for 2 h in glucose (12 mM)–histidine (4 mM)–ethanol (170 mM) solution. After 2 h 2 mM homoserine was added. The amino acid pools were sampled and analysed at intervals of 2 h. After 2 h in glucose–histidine–ethanol solution no homoserine, threonine or *O*-ethylhomoserine could be detected on chromatograms. Within 2 h of the addition of homoserine all three amino acids were found and the proportion of homoserine converted to the ether was increased. The necessary enzymes were, therefore, produced in response to methionine deficiency and not to the presence of homoserine.

Evidence for Methionine Biosynthesis

Amino acid pools were analysed automatically with plants in glucose–cysteine suspension and methionine was found to decline from the time of harvesting to undetectable concentration and then subsequently to increase to an approximate internal concentration of 50 μM. Accurate measurement of such low levels was not possible and if the development of plants was limited by the availability of methionine then it was expected that methionine would not accumulate above the minimum concentration which would support growth. An indirect approach was therefore adopted.

Methionine was shown to be required for branching in suspensions by suspending young, unbranched plants in glucose (12 mM)–histidine (4 mM) or glucose (12 mM)–leucine (4 mM) with additions of methionine to give final concentrations of 0, 25, 50, 100, 200, 400 μM. While 25 μM increased branching it required 50 μM to achieve single branches on all plants. With 100 μM methionine the tips developed 3–4 branches. Although 200 and 400 μM was initially inhibitory, multiple branching was observed later. The time of branching was 2–3 h with histidine and 5 h with leucine. The time difference relates to the differing inhibition of hyphal development by the two amino acids. The requirement of methionine was related to the concentration available at the time of harvesting. A culture grown with 600 μM instead of 300 μM methionine required only the addition of 25 μM to permit all plants to undergo single branching.

A combination of glucose (12 mM), homoserine (2 mM) and threonine (2 mM) was supplied to a suspension of plants (0·3 mg/ml, dry wt) which failed to make any detectable *O*-ethylhomoserine. The threonine was supplied to inhibit conversion of homoserine to threonine and maximize homoserine–methionine conversion. The plants in this experiment underwent multiple branching and hyphal elongation from 7 h. This result is compatible with a slow biosynthesis of methionine but does not exclude the possibility that branching is related to an intermediate of biosynthesis which is also produced by catabolism of methionine.

Cystathionine is a known intermediate of methionine biosynthesis in other organisms. It has been detected in amino acid pools of *A. macrogynus* by automatic analysis after exposure of plants to glucose with homoserine, cysteine or 4 mM methionine. In yeast the methionine biosynthetic pathway is reversible (Lawrence and Cole 1968).

Discussion

A limited range of microorganisms was shown to make *O*-ethylhomoserine when supplied with ethanol and homoserine (Murooka and Harada 1967, 1968). The enzyme involved was shown to be *O*-acetylhomoserine sulfahydrylase, an enzyme of the biosynthetic pathway to methionine. It was not clear whether the reaction with alcohols had any normal function in the organisms (Murooka *et al.* 1977). It is possible, for example, to postulate that the enzyme catalyses the elimination of acetic acid and that the intermediate then adds any available thiol or alcohol. The formation of this ether in the amino acid pools of *A. macrogynus* links two of the essential requirements for the selective development of resistant sporangia—reduced aeration and methionine deficiency (Youatt 1982*a*).

Methionine has long been considered to be an absolute requisite for the growth of *A. macrogynus* (Machlis 1953) though Nielsen (personal communication) reported that intermediates of methionine biosynthesis such as cystathionine had some ability to replace it. These and present observations suggest that the pathway may be available but not with sufficient capacity to support optimum growth.

The production of resistant sporangia correlates with the ability of amino acids to inhibit the production of zoosporangia (Sandars and Youatt 1983) with the production of *O*-ethylhomoserine and with the presence of trehalose (Youatt 1982*c*). The question therefore arose as to why suspensions supplied with glucose and *O*-ethylhomoserine did

not produce resistant sporangia sooner than 8–9 h since adequate concentrations of the ether and trehalose were already present at 2 h. To the present time 25 different amino acids have been used in combination with glucose as suspension media for *A. macrogynus*. [Aspartic and glutamic acids are not accumulated well (Sandars and Youatt 1983) and must always be considered separately.]

The amino acids which are favourable for resistant sporangium formation allow early hyphal elongation and all the plants achieve the same size when the elongation ceases regardless of the nature or concentration of the amino acid. This implies that the development is limited by a specific deficiency common to all conditions and not to variations in the availability of amino acids as nitrogen sources. In the present context it seems reasonable to suggest that hyphal elongation continues until the available methionine reaches an inadequate concentration to support further development.

It is now possible to describe a system for the control of the development of sporangia which is consistent with all experimental observations made to date. In the absence of any amino acids mRNA is rapidly transcribed for the production of zoosporangia. The prevention of this transcription by the supply of single amino acids requires rapid accumulation of the amino acid and is probably best explained by the enzymic production of ammonia at an appropriate site (Sandars and Youatt 1983). If glucose is available the trehalose-synthesizing enzymes are activated by amino acid deficiency and the trehalose content begins to rise even in the presence of cycloheximide (Youatt 1982*b*). One way of envisaging this activation is by protease action since amino acid deficiency does not prevent the maintenance of amino acid pools and protein turnover was demonstrated by Burke *et al.* (1972) in similar conditions. The protein content also declined (Youatt 1980*a*).

It is suggested that protein turnover allows hyphal extension to continue until limited by methionine deficiency. Meanwhile in the absence of external methionine the enzymes for methionine biosynthesis are synthesized. In conditions of reduced aeration the accumulation of ethanol allows *O*-ethylhomoserine to form. There are two possible ways in which the biosynthesis of methionine is then controlled. One is that the removal of homoserine from the pathway as the more stable ether prevents the biosynthesis of methionine and the other is that the ether may be an inhibitor of the *O*-acetylhomoserine sulfhydrylase. Either way the postulated role of the production of *O*-ethylhomoserine is to reduce the synthesis of methionine.

The completion of resistant sporangia requires the continuing presence of glucose until the acid-resistant wall has been completed (Youatt 1982*c*). The presence of trehalose may therefore have an indicator role that glucose is still available. Whereas glycogen synthesis continued until glucose was exhausted, trehalose synthesis ceased at about 1·5 mM and trehalose was released to the medium (Youatt 1982*c*).

The previously stated requirement that the controlling amino acid must not be metabolized before 5 h can now be restated that the controlling amino acid must be present until maximum hyphal elongation has occurred and possibly until methionine has reached a critical concentration.

The control of the transcription of mRNA for the resistant sporangium is more complicated than the control for zoosporangium production since there appears to be a simultaneous requirement for adequate glucose and low methionine. This is also in agreement with the conditions for selective production in culture conditions.

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References

Burke, D. J., Seale, T. W., and McCarthy, B. J. (1972). Protein and ribonucleic acid synthesis during the diploid life cycle of *Allomyces arbuscula*. *J. Bacteriol.* **110**, 1065–72.

Gray, W. R. (1967). End group analysis—Dansyl chloride procedure. *Methods Enzymol.* **11**, 139–51.

Lawrence, W. C., and Cole, E. R. (1968). Yeast sulfur metabolism and the formation of hydrogen sulfide in brewery fermentations. *Wallerstein Lab. Comm.* **31**, 95–115.

Machlis, L. (1953). Growth and nutrition of water molds in the subgenus Euallomyces. *Am. J. Bot.* **40**, 450–60.

Murooka, Y., and Harada, T. (1967). New amino acids: *O*-ethyl-, *O*-propyl- and *O*-butylhomoserine formed from alcohols by a soil bacterium. *Agric. Biol. Chem.* **31**, 1035–9.

Murooka, Y., and Harada, T. (1968). Formation of *O*-ethylhomoserine by bacteria. *J. Bacteriol.* **96**, 314–17.

Murooka, Y., Kakihara, K., Miwa, T., Seto, K., and Harada, T. (1977). *O*-Alkylhomoserine synthesis catalysed by *O*-acetylhomoserine sulfhydrylase in microorganisms. *J. Bacteriol.* **130**, 62–73.

Sandars, S., and Youatt, J. (1983). Amino acids in the control of differentiation of sporangia in *Allomyces macrogynus*. *Aust. J. Biol. Sci.* **36**, 435–43.

Terenzi, H. F., and Storck, R. (1969). Stimulation of fermentation and yeast-like morphogenesis in *Mucor rouxii* by phenethyl alcohol. *J. Bacteriol.* **97**, 1248–61.

Youatt, J. (1980*a*). Degradation of nucleic acid by *Allomyces macrogynus* during the production of zoosporangia and resistant sporangia. *Aust. J. Biol. Sci.* **33**, 393–401.

Youatt, J. (1980*b*). Changes in carbohydrates of *Allomyces macrogynus* during the selective development of either zoosporangia or resistant sporangia. *Aust. J. Biol. Sci.* **33**, 505–11.

Youatt, J. (1982*a*). Selective development of resistant sporangia in growing cultures of *Allomyces macrogynus* and *A. arbuscula*. *Aust. J. Biol. Sci.* **35**, 333–42.

Youatt, J. (1982*b*). Oxine, ferric oxine and copper oxine as inhibitors of growth and differentiation of *Allomyces macrogynus*. *Aust. J. Biol. Sci.* **35**, 565–71.

Youatt, J. (1982*c*). Role of glucose and amino acids in the production of resistant sporangia by *Allomyces macrogynus*. *Aust. J. Biol. Sci.* **35**, 557–63.

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