

Production of Ergot Alkaloids *in vitro* by *Sphacelia sorghi*

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SUMMARY

A method is described for the production of dihydrogenated ergot alkaloids, principally dihydroergosine, in yields exceeding 0.5 mg/ml in surface liquid cultures of *Sphacelia sorghi*. Alkaloid production occurred at the end of the growth phase and most of the product was released into the medium. An increase in the proportion of unsaturated fatty acids in the mycelial triglyceride oils during the growth phase was associated with the development of sclerotium-like tissues within the mycelial mat. Conversion of asparagine in the medium to aspartic acid by a non-alkaloid-producing strain indicated asparaginase activity. Inorganic phosphate was rapidly absorbed from the medium, and sucrose was utilized through hydrolysis followed by the preferential uptake of glucose. A glucan accumulated during growth of the alkaloid-producing strain, thus precluding a shake-culture process, whereas the small amount which was produced by a non-alkaloid-producing strain disappeared towards the end of the fermentation.

INTRODUCTION

The alkaloid components of the sclerotia of *Sphacelia sorghi* McRae, a fungus of Nigerian origin, parasitic on *Sorghum vulgare* Pers., have been described (Mantle, 1968; Mantle & Waight, 1968). The principal alkaloid was dihydroergosine, which occurs in only this ergot fungus, together with small amounts of festuclavine, pyroclavine, dihydroelymoclavine and chanoclavine. Although the natural sclerotia contained approximately 0.5% (w/w) total alkaloid, the weak pharmacological properties of dihydroergosine resulted in the sclerotia having low toxicity to mice. The method for alkaloid production *in vitro* by this organism, here reported, will now permit studies on the biosynthesis of a dihydrogenated ergot alkaloid. It will also provide, through hydrolysis of dihydroergosine, a source of dihydrolysergic acid. This is a preferred starting material for the chemical synthesis of D-6-methyl-8-cyanomethylergoline which is an anti-implantation agent for rats and mice (Rezabek, Semonsky & Kucharczyk, 1969; Mantle & Finn, 1971).

METHODS

Origin of Sphacelia sorghi strains used

The alkaloid-producing strain, designated strain A, was selected over a period of two years by screening, on the basis of alkaloid yield *in vitro*, subcultures derived from hyphal fragments and spores. The original isolate was from a sclerotium of Nigerian origin. The non-alkaloid-producing strain NA was also selected during the screening programme.

Media and culture conditions

Cultures of *Sphacelia sorghi* were maintained by subculturing every 3 weeks to slopes of sucrose/asparagine agar (Castagnoli & Mantle, 1966). Modification of the medium for

optimum alkaloid yield in liquid culture resulted in the following defined composition: sucrose, 150 g; L-asparagine, 15 g; KH_2PO_4 , 0.25 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.033 g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.027 g; distilled water, 1 l; pH 5.5, adjusted with NaOH. Erlenmeyer flasks (500 ml), containing 100 ml medium sterilized at 106 °C for 20 min, were inoculated with 1 to 2 ml of a concentrated suspension of hyphal fragments and conidia obtained by homogenizing mycelium from agar cultures. Only a small proportion of the inoculum floated to the surface, thus initiating surface growth, but it was found that the amount of 2-week-old mycelium which could conveniently be removed from a test-tube culture was sufficient when homogenized to inoculate two flasks. Flask cultures were incubated stationarily at 27 °C in the dark. The course of fermentation was followed for 26 days by removing triplicate flasks at intervals, separating the surface mycelium from the culture filtrate and measuring several parameters by the following analytical techniques.

Mycelial dry weight. The underside of mycelial mats was washed with distilled water, the mats homogenized in distilled water and freeze-dried to constant weight at approx. 20 μm mercury.

Polysaccharide. A small volume of culture filtrate was stirred with an equal volume of absolute ethanol. The presence of polysaccharide was indicated by a characteristic precipitate.

Alkaloid in culture filtrate. Culture filtrate (5 ml) was mixed directly with 10 ml van Urk reagent (0.125 % *p*-dimethylaminobenzaldehyde in 11.5 M- H_2SO_4 containing 0.1 % (v/v) of a 5 % (w/v) FeCl_3 solution). The development of a blue colour indicated the presence of ergot alkaloids and the extinction was determined colorimetrically over the linear range of 10 to 50 μg dihydroergosine/ml and compared with a standard curve. Filtrates containing more than 50 μg total alkaloid/ml were suitably diluted with distilled water before one part of diluted solution was mixed with two parts of reagent. Extraction of culture filtrate at pH 8.5 three times with an equal vol. of diethyl ether gave quantitative transfer of alkaloids, without excessive difficulty with emulsions due to the increased (glucan) viscosity of culture filtrate. The relative proportions of the component alkaloids were assessed by t.l.c. (Mantle, 1968) of the ether extract.

Alkaloid in mycelial tissue. Total alkaloid was extracted from freeze-dried mycelium, and quantitatively determined, as previously described for natural sclerotial tissue (Mantle & Waight, 1968). The relative proportions of component alkaloids were assessed as for those extracted from culture filtrate.

Mycelial lipid. Freeze-dried mycelium (up to 4 g) was extracted twice by stirring overnight at 30 °C with 100 ml chloroform:methanol (2:1, v/v). The chloroform layer containing the lipid extract was separated after partition from the methanol by addition of 0.2 vol. distilled water, and was then evaporated to dryness. The oily residue usually contained a particulate component which was separated by dissolving the oil in ether and decanting the clear supernatant. The ether extract was dried to constant wt at room temperature *in vacuo* and the lipid content of the mycelium was expressed as a percentage of the freeze-dry wt.

A portion of the oil was saponified in methanolic KOH and the resultant fatty acids methylated with diazomethane. The proportions of methyl esters of fatty acids were determined by g.l.c. using a column containing 10 % diethyleneglycol succinate on Chromosorb G operating at 195 °C, with argon as the carrier gas and detection by flame ionization.

Sugars in culture filtrate. The total sugar content of culture filtrate was determined by an automated system, developed in the Biochemistry Department, Imperial College, involving estimation of reducing sugars after acid hydrolysis. Qualitative determination of component

sugars was made by descending paper chromatography (Whatman no. 1) in *n*-propanol + ethyl acetate + water (7:1:2, by vol.) for 40 h.

Total nitrogen in culture filtrate. After automated Kjeldahl digestion, direct colorimetric determination of ammonia was made by a phenol-hypochlorite system (*Technicon Bulletin* no. 3a, Technicon Instrument Co. Ltd, Basingstoke, Hampshire).

Inorganic phosphate in culture filtrate. Automated estimations were made according to *Technicon Bulletin* no. 4b.

Free amino acids. Freeze-dried tissue was extracted with *m*-perchloric acid at 0 °C. The total amino acids were separated from other components of the perchlorate extract by cation-exchange chromatography [Zeocarb 225(H⁺)] and then autoanalysed (Thomas, 1969).

Amino acids in the culture filtrate were autoanalysed directly after suitable dilution of filtrate with 0.025 M-HCl.

RESULTS

Preliminary studies

During a series of preliminary studies in which a non-alkaloid-producing strain was selected, alkaloid production by another strain was improved by strain selection and medium development. *Sphacelia sorghi* did not utilize nitrate nitrogen, but *L*-asparagine and ammonium salts of malic, fumaric and succinic acids supported alkaloid production at an optimum temperature of 27 °C and an initial pH value of 5.5. The best carbon sources were sucrose or glucose but growth on these sugars also involved the formation of a glucan which, although less troublesome in surface cultures, rendered submerged culture difficult by increasing the viscosity of the broth and consequently impairing oxygen transfer. Zinc was an essential ingredient of the medium and the presence of iron was found to be beneficial. The initial concentration of inorganic phosphate was critical (0.025 % KH₂PO₄) in obtaining a maximal yield of alkaloid. Dry weight progressively increased as the initial concentration of KH₂PO₄ was increased up to 0.1 %, at which concentration no alkaloid was produced. Phosphate was always totally absorbed from the medium. When the optimum medium, in which asparagine was the nitrogen source, was supplemented (0.4 %, w/v) at the time of inoculation with asparagine or *L*-proline, there was little effect on alkaloid production and the control flasks yielded approx. 250 µg/ml. However, addition of *L*-tryptophan or *L*-leucine, both of which are components of the dihydroergosine molecule, completely suppressed alkaloid production, although at least 90 % of the added amino acid was found in the culture filtrate at the end of the fermentation.

Course of fermentation of Sphacelia sorghi

Alkaloid-producing strain

The progress of the fermentation process, during which a high yield of alkaloid was produced, was followed and compared with a strain which failed to yield alkaloid under the same conditions.

Strain A produced a thin white mycelium on the surface of the medium within 5 days of inoculation and extracellular glucan was also present. The glucan increased and persisted throughout the fermentation. Sporulation (both micro- and macrospores) was observed within 9 days and continued during the growth of the surface mat. Preparations stained with Sudan III showed the spores to be rich in lipid while the mycelial cells appeared to contain relatively less lipid. Dry weight reached a maximum (3.6 g from 100 ml medium) within 14 days and the surface mat became off-white, possibly owing to sporulation.

Table 1. *Fatty acid composition of triglyceride oils from natural sclerotia of Sphacelia sorghi, and from the mycelia of two strains at stages during growth in surface culture*

Strain	Days after inoculation	Fatty acid methyl esters (% molar basis)				
		16:0	18:0	18:1	18:2	18:3
A	5	80.3	17.4	2.1	0	0
	7	69.5	15.1	15.4	0	0
	9	69.7	20.4	9.9	0	0
	12	59.0	15.4	23.0	2.6	0
	14	67.0	17.5	14.2	1.3	0
	16	47.4	14.2	23.7	10.5	4.2
	20	51.4	9.6	24.2	11.2	3.6
	22	65.2	10.9	15.5	6.0	2.4
	26	44.7	9.3	32.3	11.3	2.4
	NA	9	79.3	15.2	3.7	1.8
14		42.7	5.6	29.3	20.9	1.5
20		42.4	5.2	28.4	18.6	5.4
26		39.9	4.9	24.2	23.1	7.9
Natural sclerotia (ex <i>Sorghum vulgare</i>)		40.5	5.3	46.6	5.4	2.2

Alkaloid synthesis occurred at the end of the growth phase as is common for many secondary metabolites. Most of the alkaloid was released into the medium but an increasing proportion was retained within the mycelium as alkaloid production proceeded. The first alkaloid detected in the culture was in the filtrate at 12 days and consisted mainly of dihydroergosine together with a small amount of chanoclavine and traces of festuclavine and dihydroelymoclavine. By day 16 the proportions of festuclavine and dihydroelymoclavine had increased to be approximately equal to chanoclavine, and thereafter the composition remained constant. Maximum yield of alkaloid (700 $\mu\text{g/ml}$), which had accumulated linearly after day 12, was reached by day 22.

Alkaloid was first detected in the mycelium on day 14. Chanoclavine and dihydroelymoclavine were rather less abundant than in the culture filtrate and pyroclavine was also present. The dihydrogenated ergot alkaloids are much more stable substances than unsaturated $\Delta 8-9$ or $\Delta 9-10$ ergoline derivatives, and thus the present analyses may be regarded as giving a reliable assessment of the alkaloid composition of mycelia and culture filtrates.

Non-alkaloid-producing strain

By comparison, strain NA was approximately 2 days slower in establishing mycelial growth on the surface of the medium, possibly because the inoculum contained very few spores. However, strain NA eventually achieved the same dry weight as strain A. Little or no sporulation occurred. Traces of glucan were detected during mid-fermentation but none was present at 26 days. The hyphae were found to contain more lipid (11 %, w/w) than strain A (5 %, w/w, including spore lipid), and there was a sharp change in the fatty-acid composition of the mycelial triglycerides within 9 to 14 days of inoculation (Table 1). Phosphate had also been totally absorbed from the medium within 14 days. At the end of the fermentation the pH value had risen to 7.0 (6.5 in strain A).

Significance of changes in mycelial lipid

The oil from the mycelium was rich in saturated fatty acids but later the proportion of unsaturated 18-carbon acids increased. This change was not so clearly observed in strain A but, nevertheless, the composition of the triglyceride oil from the 26-day sample most

Table 2. Changes in amino acid composition of culture filtrates during 26 days of growth of *Sphacelia sorghi* strains A and NA in surface culture

Strain	Days after inoculation	Total amino acids ($\mu\text{mol/ml}$)	%, μmolar basis			
			Asp	Asn	Glu	Gln
A	0	92.1	10.6	89.4	0	0
	5	116.4	6.5	93.5	0	0
	7	104.5	2.4	97.6	0	0
	9	88.4	1.6	98.3	0	0
	12	72.6	11.2	88.8	0	0
	14	71.7	0.5	98.6	0.9	0
	16	69.4	0.5	98.8	0.7	0
	20	60.4	0.4	98.0	1.6	0
	22	52.5	0.5	95.2	4.3	0
	26	44.2	1.0	87.9	11.0	0
	NA	9	75.9	53.6	35.8	10.6
14		*	64.5	31.5	4.0	0
20		14.7	96.9	0	3.1	0
26		25.2	35.6	0	47.4	17.0

* Not determined.

closely resembled that of natural sclerotia. However, the ratio of saturated fatty acids to unsaturated acids of natural sclerotia was more nearly reproduced in the 26-day sample of strain NA. The natural sclerotial tissues included a distinctive sphacelial portion situated at the distal end. Thus if the sphacelial (sporulating) and sclerotial tissues contained different types of triglyceride oil the values obtained for the natural sclerotial oil would depend on the proportions of these tissues. It was concluded that the triglyceride oil of conidia and young, actively growing, sphacelial hyphae of *Sphacelia sorghi* was rich in saturated fatty acids, and that sclerotial tissue contained an oil which was relatively richer in unsaturated 18-carbon acids.

Amino acids in culture filtrate

The distinctive difference between the changes in free amino acids in the culture filtrate of the two strains of *Sphacelia sorghi* (Table 2) suggested either that, by comparison with strain NA, strain A possessed very little asparaginase activity, or that it exhibited a strong preference for aspartic acid as a nitrogen source. The presence of glutamic acid throughout the fermentation of strain NA and the appearance of glutamine at the end of the growth phase was also notable. Traces of other amino acids were also usually present. Both strains similarly depleted the nitrogen content to about 1 mg N/ml.

Mycelial amino acids

The concentrations of the principal free amino acids in the mycelium presented a complex pattern but, as well as some differences, there were several features which were common to both strains. Arginine accumulated to become the principal amino acid (approx. $5 \mu\text{mol}/100 \text{ mg}$ dry mycelium) but this occurred several days earlier in strain NA. Glutamic acid increased during the fermentation, but was twice as abundant in strain NA ($1.4 \mu\text{mol}/100 \text{ mg}$ dry mycelium). Reduction in mycelial glutamine concentration in strain NA after day 20 coincided with the appearance of this substance in the culture filtrate. Two unidentified ninhydrin-positive substances, having a retention time on the amino acid autoanalyser intermediate between methionine and tyrosine, were present in both strains but reached maximum concentration much earlier in strain NA.

Utilization of sucrose

Paper chromatography of cultures filtrates showed that sucrose was rapidly hydrolysed to glucose and fructose, accompanied by a temporary accumulation of an unidentified disaccharide. Glucose was utilized preferentially and thus the principal sugar remaining at the end of the fermentation was fructose (2 to 3 %, w/v).

DISCUSSION

A fermentation process for the production of ergot alkaloids by *Sphacelia sorghi* has not previously been reported and thus it is not possible to make direct comparison with other studies. However, in view of the similarity of the imperfect fructification of this fungus with that of several *Claviceps* spp. and also the recently observed sexual stage, on the basis of which *S. sorghi* is to be described as a new *Claviceps* species (R. L. Mower, personal communication), some fermentation parameters of *S. sorghi* are compared with those of other alkaloid-producing ergot fungi.

The millet ergot fungus (*Claviceps fusiformis*) produces a conidial fructification in the early stages of growth in liquid media but alkaloid is produced principally by non-sporulating hyphae which arise later in the fermentation and are composed, like natural sclerotia, of uninucleate cells containing more triglyceride oil than do sphacelial cells (Mantle & Szczyrbak, 1972). It is possible that sphacelial hyphae can synthesize alkaloids and thus there is not such an absolute correlation between alkaloid synthesis and sclerotium-like tissue as occurs in *C. purpurea* (Mantle & Tonolo, 1968). In the present studies *Sphacelia sorghi* similarly produced a conidial fructification on cultures which subsequently synthesized alkaloid, but the morphology of hyphae in the mycelial material did not show as distinctive a contrast between thin sphacelial hyphae and broader, more frequently septate, sclerotial hyphae as occurs in *C. fusiformis*. Large differences in the composition of the triglyceride oil of sphacelial and sclerotial tissues of *C. purpurea*, *C. sulcata* and *C. fusiformis* have already been demonstrated (Mantle, Morris & Hall, 1969; Mantle, 1972; Mantle & Szczyrbak, 1972) and thus the change in the composition of *S. sorghi* triglyceride oil during growth suggests that both strains produce sclerotial tissue similar to that present in the natural sclerotium.

The accumulation of glucan in media containing sucrose or glucose is a feature of all alkaloid-producing isolates of *Sphacelia sorghi* which have been tested, but there was only a transitory occurrence in strain NA in which the growth form was considered to be more completely sclerotium-like. This may be similar to an instance described for *Claviceps fusiformis* (Dickerson, Mantle & Szczyrbak, 1970) in which the glucan synthesized by sphacelial tissue was hydrolysed through the action of a β -glucanase, which was first detected, and subsequently increased, as sclerotial tissue developed.

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