PRODUCTION OF BIKAVERIN BY *FUSARIUM OXYSPORUM* AND ITS IDENTITY WITH LYCOPERSIN*

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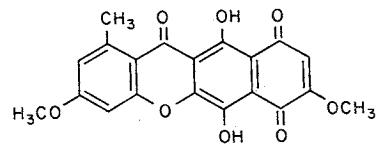
In a study of fungi in pastures associated with ovine ill-thrift[1] *Fusarium oxysporum* has frequently been encountered. This species of fungus is notable for the characteristic purple pigmentation of surface mycelium on malt agar, but was found to produce pigment irregularly in shaken cultures in a variety of media. From each of 6 producing strains the same deep red pigment giving insoluble purple salts was isolated. It resembled in most properties the red pigment lycopersin isolated in 1949 by KREITMAN and colleagues[2,3] from cultures of *F. lycopersici* and *F. vasinfectum*, two species included by SNYDER and HANSEN[4,5] within the species *F. oxysporum* SCHLECHT. Direct comparison of our pigment with an authentic specimen of lycopersin established that they were, indeed, identical.

We have also observed that the mycelium of *Gibberella fujikuroi* (SAWADA) ITO ACC 917 grown in a sucrose-soybean meal medium[6] contains small amounts of lycopersin. The presence of a wine-red antiprotozoal metabolite in cultures of *G. fujikuroi* (*F. moniliforme*) and another *Fusarium* species was first described in 1970 by BALAN and coworkers.[7] Subsequently, KJAER, BU'LOCK and their collaborators,[8] after extensive chemical investigations, have identified this substance with bikaverin, a fungal vacuolation factor discovered by PARK and coworkers[9] in ageing cultures of *F. oxysporum*



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and *F. oxysporum* f. sp. *lycopersici*. Chemical[8,10] and X-ray crystallographic[11] evidence established the structure of bikaverin as I. Its resemblance to, and despite some discrepancies in the reported properties, its probable identity with lycopersin was noted.[8,10] We have compared samples of lycopersin and bikaverin directly and confirmed their identity. As recommended by CORNFORTH and colleagues[10] the pigment will be referred to hereafter as bikaverin to avoid confusion with the carotenoid substance, lycopersene. Bikaverin has recently been reported[12] to be identical to mycogonin, the red pigment of *Mycogone jaapii* LINDAU, and is also identical to passiflorin, a pigment from *F. oxysporum* f. sp. *passiflorae*, the structure of which was reported by HOWELL and HUSTON in 1968.[13]

In studies of pigmentation in still cultures of *F. lycopersici* and *F. vasinfectum* SEBEK[14] noted that RAULIN-THOM medium, which contains nitrogen as ammonium salts, was suitable whereas CZAPEK-DOX medium with sodium nitrate as the nitrogen source was unsatisfactory. Our studies with shaken cultures have given similar results and shown that ammonium tartrate, which allows the pH of the cultures to fall to a value close to 3, is superior to other ammonium salts and to most amino acids. Only L-alanine, which promoted similarly acidic broths, supported a comparable yield of bikaverin. By adjusting the acidity of RAULIN-

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THOM medium SEBEK concluded that optimum pigmentation occurred within the pH range 3.2 ~3.7. In shaken cultures the range was slightly lower (pH 2.8~3.0) but attempts to buffer the medium with high concentrations of phosphate gave much reduced yields, apparently because of the suppressive effect of potassium phosphate (Table 1).

Bikaverin was produced to different extents when D-glucose in the glucose-ammonium tartrate medium was replaced with a variety of carbohydrates. In general our results agreed with SEBEK's qualitative observations: hexose sugars supported the highest production; only sucrose was superior to glucose with a 25 % increment in maximum attainable yield.

Table 1. Influence of potassium phosphate concentration*.

KH_2PO_4 g/liter	Mycelium g/liter	pH	Bikaverin mg/liter
0.25	15.7	3.1	1,100
0.5	16.0	2.9	891
1.0	16.4	2.8	640
2.0	16.6	3.0	501
4.0	16.9	3.1	400
8.0	17.0	3.3	222

* *F. oxysporum* was grown from a spore inoculum on a medium containing D-glucose (6 %), ammonium tartrate (0.46 %) and salts. The results shown are for cultures harvested at 7 days.

Table 2. Influence of glucose and ammonium tartrate concentration* on the yield (mg/liter) of a) bikaverin and b) mycelial lipid.

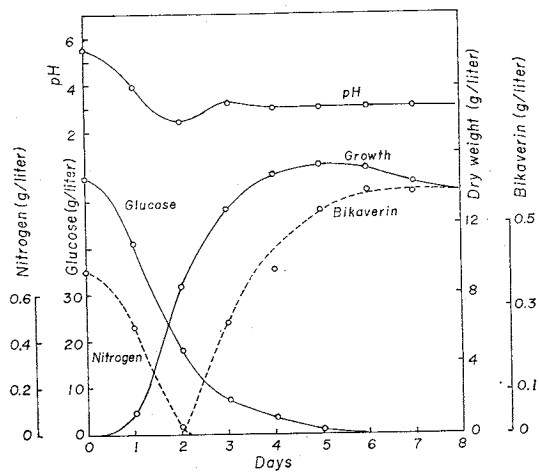
Ammonium tartrate %		D-Glucose (%)			
		1.5	3	6	12
0.12	a)	146	189	234	275
	b)	110	111	341	723
0.23	a)	104	242	421	471
	b)	62	148	725	236
0.46	a)	13	191	672	511
	b)	24	30	350	200
0.92	a)	0	0	0	0
	b)	10	10	9	113

* *F. oxysporum* was grown from a spore inoculum in a medium containing 0.1 % potassium dihydrogen phosphate and salts. Values given are for cultures harvested at 7 days.

However, the kind of carbon source used was less critical than the initial carbon : nitrogen ratio in the medium (Table 2). With increasing amounts of ammonium tartrate a proportionate increase in glucose was required to ensure heavy pigmentation. The increased yield of bikaverin with increasing initial nitrogen concentration, up to the limiting value in the region of 0.7 g (0.46 % ammonium tartrate) per liter, was correlated with increased cell yield. With an initial nitrogen concentration of 1.4 g per liter, unused ammonium ions remained at the end of the growth phase and bikaverin was not produced. However, if mycelium from such unpigmented cultures was replaced to a 6 % D-glucose solution, bikaverin was formed abundantly after a 2~3-hour lag.

These results, together with those from analysis of cultures during development (Fig. 1) indicate that bikaverin is synthesized only during unbalanced growth in acidic media when the nitrogen source has been depleted and excess carbon source remains. Enhanced pigmentation in *Fusarium* species cultured on media

Fig. 1. Accumulation of mycelium and bikaverin, changes in pH, and utilization of glucose and ammonia, during growth of *F. oxysporum* from a spore inoculum on a glucose (6 %), ammonium tartrate (0.46 %), potassium dihydrogen phosphate (0.1 %), salts medium.



rich in glucose has been noted by earlier workers.[15,16] NORD and colleagues[16] also observed a strong correlation between pigmentation and accumulation of lipid in *F. lycopersici*. An analysis of the bikaverin and lipid content of *F. oxysporum* grown in media varying in initial carbon : nitrogen ratio supported this observation but showed that the two processes do not respond identically to changes in the nitrogen balance of the medium (Table 2). Synthesis of excess lipid required a higher carbon : nitrogen ratio, and persisted longer in nitrogen-depleted cultures, than did the synthesis of bikaverin. The precise amounts of carbon and nitrogen sources required initially in the medium to insure abundant pigment production varied with growth conditions and inoculum size. Thus the concentrations established for a small spore inoculum (Table 2) were suboptimal when a large vegetative inoculum was used. High and reproducible yields of bikaverin were then obtained by raising the glucose concentration to 12 %.

Experimental

Culture of *F. oxysporum*: Cultures were maintained on 2 % malt agar. A spore inoculum was prepared by transferring a piece of mycelium to a 125-ml Erlenmeyer flask containing 50 ml of the following medium: D-glucose (2 %), ammonium tartrate (0.46 %), potassium dihydrogen phosphate (0.1 %), magnesium sulfate heptahydrate (0.05 %), sodium chloride (0.01 %), calcium chloride (0.01 %) and 1 % (v/v) of a trace mineral solution containing cupric sulfate pentahydrate (393 mg), boric acid (57 mg), ammonium molybdate tetrahydrate (37 mg), manganese sulfate monohydrate (61 mg), zinc sulfate heptahydrate (8.79 g) and ferrous sulfate heptahydrate (1 g) in 1 liter of water. The culture was incubated at 25°C for 3 days on a rotary shaker with a speed of 220 r.p.m. and eccentricity of 1.5 inch (3.8 cm), then filtered through sterile cotton. A portion (1 ml) of the filtered spore suspension, adjusted to give a concentration of 2×10^7 spores per ml, was used to inoculate each 50 ml of production medium. Production cultures were grown under the same conditions as the inoculum, except for variations in the carbon source, nitrogen source, or potassium dihydrogen phosphate content of the medium. Adjustments of pH were made with N-hydrochloric acid or sodium hydroxide.

Analyses: Production cultures grew as an evenly dispersed suspension of mycelium. Three replicates were analyzed at each sampling. Bikaverin was estimated in a 1-ml aliquot by diluting with 9 ml of glacial acetic acid, heating at 100°C for 2 minutes, clarifying by centrifugation and measuring the optical density at 500 nm. Thin-layer chromatography of culture extracts showed bikaverin to be the only colored substance present. For the estimation of lipid, 10-ml aliquots of the culture were mixed with 10 ml of 4 N hydrochloric acid. After 1 hour the mycelium was collected, washed free of acid, dried at 40°C *in vacuo*, and extracted for 48 hours with petroleum ether (bp 35~60°C). Solvent was evaporated from the extract and the residue weighed. Growth was measured as the dry weight (105°C for 16 hours) of mycelium recovered by filtration from an aliquot of the culture. The filtrate was analyzed for glucose by SOMOGYI's method,[17] and for ammonia by titrating the distillate obtained after heating with magnesium oxide.

Isolation of bikaverin from *F. oxysporum*: Mycelium from 44 liters of cultures grown for 7 days on a medium containing 6 % glucose, 0.46 % ammonium nitrate, 0.1 % potassium dihydrogen phosphate, and basal salts, was macerated with 5 liters of 0.1 N hydrochloric acid, washed thoroughly with water, and extracted with acetone for 36 hours in a Soxhlet apparatus. The evaporated extract was leached with *n*-hexane to remove lipid, then with chloroform (8×500 ml) to recover bikaverin. The chloroform solution was filtered through a shallow bed (1 cm high, 15 cm diameter) of silicic acid, reduced to half volume, and shaken with 5 % aqueous sodium bicarbonate (3×1.3 liter). The insoluble sodium salt of bikaverin recovered from the interface was suspended in 0.1 N hydrochloric acid (1 liter) and reextracted into chloroform (4×1 liter). The chloroform solution was dried (anhydrous sodium sulfate) and concentrated to 200 ml, when bikaverin (3.4 g) crystallized as deep red blades, m.p.

325~335°C (decomp.). This product was a solvate conforming approximately to the composition $C_{20}H_{14}O_8 \cdot 1/3\ CHCl_3$ (Found: C, 57.27; H, 3.45; Cl, 7.25 %). After heating at 75°C *in vacuo*, or recrystallizing as fine red needles by extracting into chloroform under reflux from a Soxhlet thimble, the unsolvated form of unchanged m.p. was obtained (Found: C, 62.72; H, 3.81 %. Calc. for $C_{20}H_{14}O_8$: C, 62.83; H, 3.69 %).

Comparison of bikaverin, lycopersin and passiflorin: A specimen of lycopersin obtained from Dr. F.F. NORD required purification. The sample (10 mg) in chloroform was filtered through a shallow bed of silicic acid and crystallized, affording 6 mg of the unsolvated form. The infrared spectrum (in KBr) was indistinguishable from those of bikaverin samples obtained by us from *F. oxysporum*, an authentic specimen of bikaverin obtained from Dr. J.W. CORNFORTH, and a sample of passiflorin obtained from Dr. W. C. HOWELL. Thin-layer chromatography of the samples on silica gel G with chloroform-methanol-acetic acid (94:1:5) gave a single violet zone, Rf 0.45.

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