ANGIOTENSIN CONVERTING ENZYME INHIBITORS PRODUCED BY *STREPTOMYCES CHROMOFUSCUS*

DISCOVERY, TAXONOMY AND FERMENTATION†

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Culture A58365.1, NRRL 15098, identified as a new strain of *Streptomyces chromofuscus*, was found to produce two novel angiotensin converting enzyme (ACE) inhibitors, A58365A and A58365B. Fermentation medium studies afforded an increase in ACE inhibitor titers from less than 1 μg/ml to greater than 20 μg/ml. Proline was the obligatory supplement for ACE inhibitor biosynthesis.

The recognition that microorganisms produce desirable metabolites depends on an efficient and simple screening procedure. An example of such a system was described in the preceding communication[1] which ultimately led to the discovery of angiotensin converting enzyme (ACE) inhibitors produced by culture A58365. In addition to the ACE inhibitors, asukamycin[2] and methylenomycin[3] are co-produced in this fermentation. ACE inhibitors have also been discovered in fermentation broths from other cultures.[4~8] The discovery that the ACE inhibitors, A58365A and A58365B, exhibited a fluorescence spectrum enabled the development of a quantitative HPLC assay procedure.[9]

This communication describes the taxonomy of the producing culture and the fermentation conditions necessary for the biosynthesis of ACE inhibitors by culture A58365. The accompanying paper describes isolation and characterization of the ACE inhibitors.[9]

Materials and Methods

Taxonomy

Culture A58365 was isolated from a soil sample collected in Brazil. Taxonomic studies identified A58365.1, NRRL 15098, a natural variant of the soil isolate, as a new strain of *Streptomyces chromofuscus*. The hydrolyzed whole cells of NRRL 15098 prepared by the method of BECKER *et al.*[10] contained LL-diaminopimelic acid, glucose, mannose, ribose and rhamnose. These components are indicative of a type I cell wall with no characteristic sugar pattern. The presence of these components is consistent with the genus *Streptomyces*.[11] The data obtained from NRRL 15098 are summarized in Tables 1 and 2. When compared with *S. chromofuscus* ATCC 23896, both cultures were found to be in the gray color series, have similar reverse colors, lack soluble pigments, possess spiral sporophores with spores of similar shape and length, have a spiny spore surface and have a similar carbon utilization pattern. These comparisons indicated that culture A58365.1 resembled ATCC 23896 with only minor differences.

A second culture, A58365.3, an *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine induced mutant of A 58365.1, was also used in this investigation.

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Table 1. Cultural characteristics of *Streptomyces chromofuscus* NRRL 15098.

Medium	Growth characteristic[a]
ISP No. 2[b]	Abundant growth; abundant aerial mycelia: 2dc yellowish gray; reverse; 69 deep OY; no soluble pigment
ISP No. 3	Fair growth; reverse: 91.d.gy.Y; poor aerial mycelial growth: 5fe light grayish reddish brownish; no soluble pigment
ISP No. 4	Abundant growth; reverse: 56. deep Br; abundant aerial mycelia: 2fe medium gray; no soluble pigment
ISP No. 5	Fair growth; reverse: 94.1.01 Br; fair aerial mycelial development: 2dc yellowish gray; light yellow soluble pigment
CZAPEK's agar	Fair growth; reverse: 79.1.gy.yBr; fair aerial mycelial development: 3ge light grayish yellowish brown; no soluble pigment
TPO[c]	Abundant growth; reverse: 75. deep yBr; abundant aerial mycelia: 3ge light grayish yellow brown; no soluble pigment

[a] The numbers and letters are used to designate color of aerial mycelia.[12] The numbers and letters used for reverse side colors refer to the color charts of ISCC-NBS Centroid Color Charts Standard Sample N. 2106, U.S. Department of Commerce, National Bureau of Standards.
[b] ISP is International Streptomyces Project media.
[c] Tomato paste oatmeal agar.

Table 2. Carbon utilization of *Streptomyces chromofuscus* NRRL 15098.

Carbon source	Utilization[a]
No carbon	−
L-Arabinose	+
D-Fructose	+
D-Glucose	+
i-Inositol	+
D-Mannitol	+
Raffinose	−
L-Rhamnose	+
Sucrose	Doubtful
D-Xylose	+
D-Arabinose	+
Cellobiose	+
D-Galactose	+
Lactose	+
D-Maltose	+
Melbiose	+
Sodium acetate	−
Sodium citrate	+
Sodium succinate	+
D-Ribose	+
Salicin	+

[a] −; No utilization, +; utilization.

Assay of ACE Inhibitor

The HPLC assay system is described in the following paper.[9] A typical fermentation profile is presented in Fig. 1. Other fluorescent peaks are evident in the tracing, the most obvious being the peak between A58365A and A58365B. This peak and the others eluting before A58365A did not appear to be associated with ACE inhibitory activity.

Fig. 1. HPLC profile of *Streptomyces chromofuscus* NRRL 15098 fermentation.

The broth supernatant was adjusted to pH 2.0 and filtered through a 0.45 μm filter.

Injection volume: 10~25 μl. Column: 4×300 mm. Resin: μBondapak C_{18}. Solvent: acetonitrile - formic acid - water (6: 0.3: 93.7). Flow rate: 2.5 ml/minute. Detection: Schoeffel model FS970 spectrofluorometer, 327 nm excitation, 370 nm cut off filter.

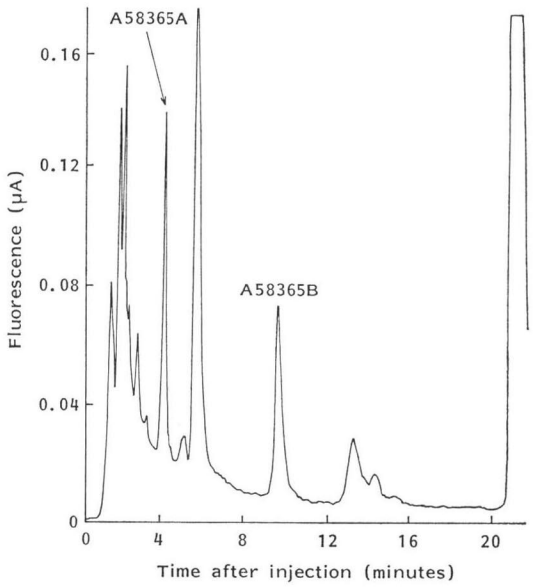


Table 3. The requirement of proline for ACE inhibitor biosynthesis by *Streptomyces chromofuscus* NRRL 15098.

Fermentation time (hours)	ACE inhibition[a]			
	Medium A		Medium B	
	—[b]	+[c]	—	+
48	0	17	10	20
72	10	20	12	18
96	10	20	12	17

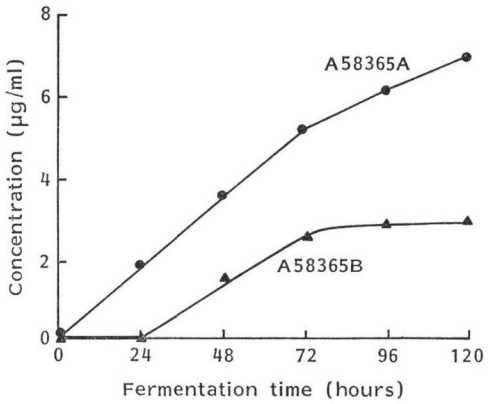
[a] Zone of inhibition (mm) on ACE agar plates. Average from duplicate samples.
[b] No proline.
[c] 0.2% proline supplementation.

Medium A contained potato dextrin 1.0%, yeast extract 0.1%, NZ-Amine A 0.2%, beef extract 0.1% and $CoCl_2 \cdot 6H_2O$ 0.001%. The pH was adjusted to 7.0.

Medium B contained glucose 2.5%, corn starch 1.0%, OM peptone (Amber Labs) 1.0%, NZ-Amine A 0.4%, black strap molasses 0.5%, $MgSO_4 \cdot 7H_2O$ 0.07%, KCl 0.02% and $FeSO_4 \cdot 7H_2O$ 0.004%. The fermentation was conducted in 250-ml Erlenmeyer flasks.

Fig. 2. The biosynthesis of A58365A and A58365B by *Streptomyces chromofuscus* NRRL 15098 in the presence of proline.

The production medium consisted of potato dextrin 3.5%, NZ-Amine A 0.4%, yeast 0.025%, beef extract 0.5% and L-proline 0.4%. At the indicated incubation times, a sample for HPLC assay was prepared as described in Fig. 1. The detection system was coupled to a Hewlett-Packard 3390 Integrator.



The ACE agar plate test was described in the preceding paper.[1]

Fermentation

A lyophilized pellet of culture A58365.1 was used to inoculate 50 ml of Trypticase soy broth containing 1% glucose in a 250-ml Erlenmeyer flask and the culture was incubated for 48 hours on a rotary shaker at 30°C (250 rpm, 6.4 cm throw). An 1% (v/v) inoculum was made from the above vegetative medium to 250-ml Erlenmeyer flasks containing 50 ml production medium and unless stated otherwise, incubated at 30°C on a rotary shaker. The flasks were harvested by centrifugation, and the supernatant was used to assay ACE inhibitor levels.

For inoculation of 100 liters of production medium in 165-liter fermentors, 10 ml of vegetative culture was used to inoculate two-liter Erlenmeyer flasks containing 400 ml Trypticase soy broth plus 1% glucose. This second stage vegetative medium was incubated at 30°C on a rotary shaker for at least 24 hours; a 0.8% (v/v) inoculum was routinely used for inoculation of the production medium. The dissolved oxygen content of the production medium was maintained at about 30 to 40% of air saturation. The pH of the medium increased from an adjusted initial pH of 7.0 to a terminal pH of 8.0~8.5 in 90~100 hours.

Results and Discussion

Initial fermentation studies were conducted in several fermentation medium formulations. Numerous attempts to increase the biosynthesis of the ACE inhibitors by supplementation of the media with the more common substrates were unsuccessful. The successful use of proline in the design and synthesis of ACE inhibitors[18] led us to supplement two media with L-proline on the assumption that this amino acid was an integral part of the A58365 compounds. The agar plate test results (Table 3) clearly demonstrated the stimulation of ACE inhibitor biosynthesis by L-proline. Initial isolation studies on the ACE inhibitors were conducted in an OM peptone (Amber Laboratories) containing fermentation medium similar to Medium B (Table 3). This ingredient was eventually eliminated to

Table 4. Effect of tyrosine and proline on A58365A biosynthesis by *Streptomyces chromofuscus* NRRL 15098.

Culture	Addition	μg/ml at 96 hours	
		A58365A	A58365B
A58365.1	0.4% L-Proline	10[a]	2
or	0.1% L-Tyrosine	1	1
A58365.3	0.4% L-Proline + 0.1% L-tyrosine	23	1

[a] Maximum ACE inhibitor titers obtained from several experiments.

Medium: Potato dextrin 3.5%, NZ-Amine B 0.4%, yeast 0.025%, beef extract 0.5%, $CoCl_2 \cdot 6H_2O$ 0.001% and deionized water.

Table 5. The requirement of proline and lysine for stimulation of A58365B biosynthesis by *Streptomyces chromofuscus* NRRL 15098.

Culture	Addition (0.2%)	μg/ml at 96 hours
		A58365B
A58365.1	None	1.2
	L-Lysine (no proline)	0.4
	L-Lysine	5.5
	D-Lysine	8.7
	DL-Lysine	6.7
A58365.3	None	0.8
	L-Lysine	6.8

Medium: Potato dextrin 2.5%, yeast 0.025%, $CoCl_2 \cdot 6H_2O$ 0.001%, beef extract 0.1%, NZ-Amine A 0.3%, L-proline 0.3% and deionized water.

Table 6. Fermentation time course of ACE inhibitor biosynthesis in 165-liter fermentors by *Streptomyces chromofuscus* NRRL 15098.

Hour	A58365A (μg/ml)[a]	A58365B (μg/ml)
0	—	—
21	4.2	—
42	13.3	2.7
66	20.6	4.8
74	22.2	4.9
90	23.1	4.4

[a] Composite data from 10 fermentation experiments.

Medium: Potato dextrin 3.5%, NZ-Amine B 0.4%, yeast 0.025%, meat peptone 0.2%, $CoCl_2 \cdot 6H_2O$ 0.001%, L-proline 0.4%, K_2HPO_4 2.5 mM and deionized water.

Aeration: 0.25 volumes of air per volume of medium per minute. Agitation: 200~250 rpm. Temperature: 29°C.

Table 7. The effect of dissolved oxygen level on A58365A biosynthesis by *Streptomyces chromofuscus* NRRL 15098.

Dissolved oxygen (% of air saturation)	μg/ml at 96 hours
	A58365A
10	5.4
20	21.2
30	22.2
40	20.8
60	14.8

The production medium is described in Table 6. Temperature: 29°C.

simplify isolation methodology and sample preparation for HPLC assay.

The requirement for proline was more precisely established when ACE inhibitor biosynthesis was quantitatively monitored by HPLC. A typical fermentation time course is presented in Fig. 2. Without proline, less than one μg/ml of both factors was synthesized. D-Proline or L-hydroxyproline could be substituted in place of L-proline but were not superior to L-proline; radioactive studies conducted with L-[*U*-^{14}C]proline suggested a direct incorporation into A58365A but not into A58365B (data not shown).

Greater amounts of A58365A were synthesized when L-tyrosine was added to the medium (Table 4). However, this regulation was evident only in combination with proline but not tyrosine alone, an expected result. For reasons unknown, the stimulation of A58365A by proline and tyrosine was not observed in 165-liter fermentors. In addition, incorporation of L-[*U*-^{14}C]tyrosine into the ACE inhibitors was not demonstrated.

The structure elucidation of A58365B[9] strongly suggested that the most obvious supplements to increase or regulate synthesis of this factor were pipecolic acid and lysine. The latter amino acid can be metabolized to pipecolate through piperideine-2-carboxylate, a widely recognized pathway.[14]

The addition of lysine to the proline supplemented medium resulted in regulating the fermentation to produce greater amounts of A58365B (Table 5); synthesis of A58365A was not significantly affected. Like the regulation of A58365A by proline and tyrosine, stimulation of the synthesis of A58365B was evident with proline and lysine but not lysine alone suggesting that A58365B synthesis is closely linked A58365A synthesis. The utility of both isomers suggest the existence of a lysine racemase(s). An unexpected result was the failure of pipecolate to stimulate A58365B synthesis. However, it is entirely possible that this culture is impermeable to this compound or that the pathway described above is not operative in NRRL 15098.

The fermentation time course in 165-liter fermentors is summarized in Table 6. As described earlier in Erlenmeyer flask studies, A58365A is synthesized before A58365B. The effect of dissolved oxygen level on biosynthesis is presented in Table 7. The indicated dissolved oxygen levels were obtained by varying the rpm and aeration parameters. To obtain the best titers for A58365A, the dissolved oxygen level was maintained at or near 30% of air saturation.

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