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 communica-
tion which ultimately led to the discovery of angiotensin converting enzyme (ACE) inhibitors produced
by culture A58365. In addition to the ACE inhibitors, asukamycin and methylenomycin are co-
produced in this fermentation. ACE inhibitors have also been discovered in fermentation broths
from other cultures. The discovery that the ACE inhibitors, A58365A and A58365B, exhibited a
fluorescence spectrum enabled the development of a quantitative HPLC assay procedure.

This communication describes the taxonomy of the producing culture and the fermentation con-
ditions necessary for the biosynthesis of ACE inhibitors by culture A58365. The accompanying paper
describes isolation and characterization of the ACE inhibitors.

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. contained L-l-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*. 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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St. Louis, MO, U.S.A.
Table 1. Cultural characteristics of *Streptomyces chromofuscus* NRRL 15098.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Growth characteristic&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISP No. 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Abundant growth; abundant aerial mycelia: 2dc yellowish gray; reverse; 69 deep OY; no soluble pigment</td>
</tr>
<tr>
<td>ISP No. 3</td>
<td>Fair growth; reverse: 91.d.gy.Y; poor aerial mycelial growth: 5fe light grayish reddish brownish; no soluble pigment</td>
</tr>
<tr>
<td>ISP No. 4</td>
<td>Abundant growth; reverse: 56. deep Br; abundant aerial mycelia: 2fe medium gray; no soluble pigment</td>
</tr>
<tr>
<td>ISP No. 5</td>
<td>Fair growth; reverse: 94.1.01 Br; fair aerial mycelial development: 2dc yellowish gray; light yellow soluble pigment</td>
</tr>
<tr>
<td>CZAPEK’s agar</td>
<td>Fair growth; reverse: 79.1.gy.yBr; fair aerial mycelial development: 3ge light grayish yellowish brown; no soluble pigment</td>
</tr>
<tr>
<td>TPO&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Abundant growth; reverse: 75. deep yBr; abundant aerial mycelia: 3ge light grayish yellow brown; no soluble pigment</td>
</tr>
</tbody>
</table>

<sup>a</sup> The numbers and letters are used to designate color of aerial mycelia.<sup>12)</sup> 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.

<sup>b</sup> ISP is International Streptomyces Project media.

<sup>c</sup> Tomato paste oatmeal agar.

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

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Utilization&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>No carbon</td>
<td>–</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>+</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>+</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>+</td>
</tr>
<tr>
<td>l-Inositol</td>
<td>+</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>+</td>
</tr>
<tr>
<td>Raffinose</td>
<td>–</td>
</tr>
<tr>
<td>L-Rhamnose</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Doubtful</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>+</td>
</tr>
<tr>
<td>D-Arabinose</td>
<td>+</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>+</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
</tr>
<tr>
<td>D-Maltose</td>
<td>+</td>
</tr>
<tr>
<td>Melbiose</td>
<td>+</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>–</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>+</td>
</tr>
<tr>
<td>Sodium succinate</td>
<td>+</td>
</tr>
<tr>
<td>D-Ribose</td>
<td>+</td>
</tr>
<tr>
<td>Salicin</td>
<td>+</td>
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</table>

<sup>a</sup> –; No utilization, +; utilization.

Assay of ACE Inhibitor

The HPLC assay system is described in the following paper.<sup>9</sup> 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 x 300 mm. Resin: µBondapak C<sub>18</sub>. 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.
The ACE agar plate test was described in the preceding paper.\textsuperscript{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 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 sooy 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.

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Fermentation time (hours) & Medium A & Medium B \tabularnewline
\hline
48 & 0 & 17 & 10 & 20 \tabularnewline
72 & 10 & 20 & 12 & 18 \tabularnewline
96 & 10 & 20 & 12 & 17 \tabularnewline
\hline
\end{tabular}
\caption{The requirement of proline for ACE inhibitor biosynthesis by \textit{Streptomyces chromofuscus} NRRL 15098.}
\end{table}

The ACE agar plate test was described in the preceding paper.\textsuperscript{13)}

\textbf{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 50 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.

\textbf{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\textsuperscript{13) 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
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-14C]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-14C]tyrosine into the ACE inhibitors was not demonstrated.

The structure elucidation of A58365B 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.\(^\text{14}\)
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 to 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.

Acknowledgments

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References

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