Lincomycin, rational selection of high producing strain and improved fermentation by amino acids supplementation

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Abstract Based on the report that the introduction of the biosynthetic precursor of lincomycin, propylproline, could increase the production of lincomycin (Bruce et al. in US Patent 3,753,859, 1973), a mutant strain pro10–20, with resistance of feedback suppression of proline (an analog of propylproline) was thus selected and lincomycin production increased by 10%. The addition of three amino acids (L-proline, L-tyrosine, L-alanine) which are the precursors of propylproline to the fermentation medium was found to enhance the accumulation of L-dopa through different pathways and was favorable to lincomycin biosynthesis. The production of lincomycin was increased by 23, 10, 13%, respectively, with the addition of 0.05 g L^{-1} L-proline at 60 h, 0.005 g L^{-1} L-tyrosine and 0.1 g L^{-1} L-alanine directly in the medium.

Keywords Lincomycin · Rational screening · Propylproline · Amino acids · Metabolic engineering

Introduction

As a lincosamide antimicrobial agent, lincomycin has gained clinical acceptance as a major antibiotics for the treatment of diseases caused by Gram-positive microbes [25]. Various efforts have been made to improve the fermentation yield of lincomycin [9, 15, 24, 26, 30]. The biosynthetic pathway and genetic control of lincomycin fermentation were described recently. The lincomycin biosynthesis proceeds via a heterogeneous rooting biphasic pathway giving rise to propylproline and methylthiolincosamide. These subunits then become condensed to N-demethyllincomycin, which is finally methylated to yield lincomycin [21].

Some major metabolic precursors of lincomycin have been isolated and identified [6, 7, 15]. Among these precursors, it has been reported that the biosynthesis of lincomycin was stimulated at low levels of propylproline [8], indicating the feasibility of improving the production of lincomycin by adjusting the levels of propylproline during fermentation. However, the exact amount of propylproline needed during different stages of fermentation is uncertain and the maximum concentration added to the culture is unknown. So the synthesis of propylproline is presumed to be the rate-limiting step in the biosynthesis of lincomycin. This hypothesis was verified in our laboratory and in this study. We developed a method using L-proline as an analog of propylproline to screen a mutant strain which can release the feedback control by propylproline to increase lincomycin production.

Three amino acids including L-proline, L-tyrosine, L-alanine were added to the medium which augmented the accumulation of dopa. The concentrations of L-tyrosine, L-dopa in both base media and fed media were carefully determined since they are important indications for the synthesis of propylproline [6, 7].

Materials and methods

Microorganisms

These studies were carried out with Streptomyces lincolnensis 55-20 which was isolated and preserved in our
Spores of S. lincolnensis were maintained in 25% glycerol at −70 °C; 0.48 mL of this suspension was used to inoculate 30 mL of a preculture medium. This medium contained (per liter): 20 g starch, 10 g glucose, 10 g soybean meal, 30 g corn steep liquor (CSL, wet weight), 1.5 g (NH₄)₂SO₄ and 5 g CaCO₃. The precultures were conducted in 250 mL Erlenmeyer flasks at 28 °C on rotary shakers at 240 rpm for 48 h. The resulting precultures were inoculated into the desired fermentation medium at an inoculum size of 0.8% vol/vol. The fermentation medium used for both mycelia growth and lincomycin production contained (per liter): 100 g glucose, 25 g soybean meal, 2 g corn steep liquor (wet weight), 8 g (NH₄)₂SO₄, 0.2 g KH₂PO₄, 8 g NaNO₃, 5 g NaCl and 8 g CaCO₃.

Three amino acids (L-proline, L-tyrosine, L-alanine) were added to the fermentation medium at different concentrations in different experiments. The pH was adjusted to 7.0–7.5 before sterilization. Cultures were conducted at 28 °C in 250-mL Erlenmeyer flasks on rotary shakers at 240 rpm for 168 h.

The soybean meal and corn steep liquor used for media preparation were supplied by Topfond Pharmaceutical Co., Henan, China.

Isolation of proline-resistant mutant pro10–20

A spore suspension of S. lincolnensis 55-20 was spread on agar plates containing 0.01, 0.05, 0.1 g L⁻¹ of proline (an analog of propylproline) and incubated at 28 °C. Colonies were picked after 7 days of incubation.

Measurement of growth

The culture samples taken at different times were centrifuged at 10,000 rpm and 4 °C for 10 min to collect the mycelia. The collected mycelia were washed three times with deionized water, centrifuged and dried at 80 °C for 24 h in a desiccator to measure the dry weight of mycelia (DMW). The measurement was done in triplicate each time.

Lincomycin determination

Lincomycin present in the culture medium was determined by an agar diffusion assay with Sarcina lutea as the test organism. Triplicate assays were done; the variation was less than 5% verified by HPLC following the procedures described in [18, 19]. The HPLC system was comprised of a Waters 1525 Binary HPLC Pump, a Waters 717plus Auto sampler, a Waters 2487 Dual wavelength Absorbance Detector, a C18 column (Agilent, 4.6 × 200 mm, 5 μm particle diameter), and a personal computer (IBM ThinkPad T40 7273-76c). The broth was centrifuged at 10,000 rpm and 4 °C for 10 min, filtered through a 0.22 μm membrane filter (Millipore Co., United States). The analysis was done under isocratic conditions. The mobile phase consisted of methanol 0.05 mol L⁻¹ sodium tetraborate (60:40, pH6.0) with a flow rate of 0.8 mL min⁻¹. The column temperature was 25 °C, the detection wavelength was 214 nm and the sample volume was 10 μL. Before each injection, the column was equilibrated for 30 min with the same mobile phase.

In this study, relative potency was used to compare the produced lincomycin. The relationship between the relative potency and actual potency is as follows:

\[ R_P = \left( \frac{A_P}{A_C} \right) \times 100 \]  

where \( R_P \) means the relative potency; \( A_P \) and \( A_C \) are the values of actually measured potency for the test experiment and control, respectively.

L-tyrosine and L-dopa determination

L-tyrosine and L-dopa in the broth were detected by HPLC according to the method described by Xu [29], originally described for levodopa in levodopa inclusion complex. The HPLC system was the same as that used for lincomycin measurement. The broth was centrifuged at 10,000 rpm for 10 min at 4 °C, then the supernatant was filtered through a 0.22 μm filter membrane. The analysis was done under isocratic conditions and the mobile phase was a solution consisted of methanol: 0.01 mol L⁻¹ KH₂PO₄ buffer solution (pH3.0) at 25:75 (v/v), with a flow rate of 0.7 mL min⁻¹. The column temperature was 30 °C, the detection wavelength was 280 nm and the sample volume was 10 μL. Before each injection, the column was equilibrated for 30 min with the mobile phase.

Pyruvic acid determination

Pyruvic acid was assayed by HPLC, the equipments were the same as those used for determination of L-dopa. The analysis was done under isocratic conditions, and the mobile phase was a NaH₂PO₄ buffer solution (0.025 mol L⁻¹) containing 1% methanol. After injection of the sample (10 μL), pyruvic acid was detected by measuring absorbance at 210 nm. The column temperature was 30 °C.
Preparation of crude, cell-free extracts

Mycelia were separated from the culture medium by centrifugation (15 min at 8,000 rpm) and were washed twice with distilled water by centrifugation under the same condition. Supernatant was filtered through a membrane filter (0.45 μm). Mycelia were disrupted in an ice-water bath with a sonicator (model JY92-II, Scientz Co., China) for 60 cycles with a working period of 3 s in each cycle of 6 s at 300 W. Cell fragments were removed by centrifugation at 10,000 rpm for 30 min at 4°C. Protein concentrations in the cell-free extract (the supernatant) were measured by the method of Lowry et al. [17] with bovine serum albumin as the standard.

Enzyme activities

These enzyme activities were determined from at least two cultures in each growth condition. For each culture, three time points were taken to establish the rate of enzymic reaction. The mean values of all these assays were calculated. The variation was no more than 5%.

Pyruvate kinase activities

Pyruvate kinase (PK) activities were measured as described by John [10] originally described for PK in blood cell. The assay was carried out at 30°C in a 1 M Tris/HCl (pH 8.0) buffer containing: 1 M KCl, 0.1 M MgCl₂, 2 mM NADH, 30 mM ADP, 60 IU LDH and 0.5 mL of crude extract or supernatant. After a 10-min preincubation at 37°C, the reaction was started by adding 30 mM PEP to the reaction mixture. One unit of enzymatic activity was defined as the quantity of enzyme that led to a variation of 1 μmol NAD⁺ per minute. The specific enzymatic activity was expressed in terms of U mg⁻¹ of protein.

Results

Isolation of proline-resistant mutant pro10–20

Colonies were picked after 7 days of incubation. The colonies on the plates containing proline were considerably different from those on the plates without proline (Fig. 1). There were no white spores on the colonies but deeper-color melanin zones around them which were presumed to be associated with oxidation of L-tyrosine and L-dopa. Colonies resistant to 0.1 g L⁻¹ of proline and with more melanin generating were picked out, and their yield of lincomycin were tested. One of the proline-resistant mutant, pro10–20, which gave the highest yield of lincomycin was chosen as a producer of lincomycin during the amino acids feeding experiments.

Proline effect on lincomycin production

Effect of initial L-proline concentrations

Several fermentations with different concentrations of L-proline (0.01, 0.05, 0.1, 0.5, 1 and 2 g L⁻¹) were carried out (Table 1). The highest lincomycin yield was observed when 0.05 g L⁻¹ of L-proline was added, the average lincomycin yield was increased by 14% compared with that of the control.

Effect of the time to add L-proline

To determine the effect of the time to add L-proline on lincomycin biosynthesis, L-proline (0.05 g L⁻¹) was added at different time and lincomycin formed are shown in Table 2. Addition of L-proline at the beginning of the culture stimulated lincomycin production by about 12%. And when proline was delivered at 60 h (the beginning of the lincomycin production phase), the total yield of lincomycin was greatly increased by 23%.

To determine whether the added proline was converted to propylproline which is considered as a precursor of lincomycin, we determined the concentrations of L-tyrosine and L-dopa which have direct correlations with the biosynthesis of propylproline. The addition of them to the culture medium has been found to induce accumulation of propylproline [6, 7].

Determination of the nature of L-proline effect

The trends of concentrations of L-tyrosine and L-dopa in the fermentation which was supplied with (0.05 g L⁻¹ at
When proline was provided in the fermentation medium, DMW increased a little during the fermentation. Compared with the effect on DMW, the presence of L-proline had a considerable effect on the accumulation of tyrosine and dopa during the lincomycin production phase (Fig. 2). The concentrations of both compounds increased in the culture with proline compared with that without proline supplementation, and this increase was more conspicuous during 60–100 h before they were used the most intensely. At the same time, mycelia dry weight accumulations were nearly the same in the two fermentations during this time. That means more tyrosine and dopa were converted from proline for the biosynthesis of propylproline.

These results indicate that proline seemed to supply as a precursor of tyrosine whose accumulation was favorable to propylproline biosynthesis.

### Table 1: Effect of increasing initial concentrations of L-proline on lincomycin production

<table>
<thead>
<tr>
<th>L-proline (g L(^{-1}))</th>
<th>0.01</th>
<th>0.05</th>
<th>0.1</th>
<th>0.5</th>
<th>1.0</th>
<th>2.0</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative potency</td>
<td>103 ± 4.50</td>
<td>114 ± 5.32</td>
<td>110 ± 3.88</td>
<td>107 ± 2.97</td>
<td>105 ± 3.98</td>
<td>102 ± 4.15</td>
<td>100</td>
</tr>
</tbody>
</table>

Values are mean ± SD of triple determinations

### Table 2: The effect on lincomycin potency with addition of 0.05 g L\(^{-1}\) L-proline at different time

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0</th>
<th>12</th>
<th>36</th>
<th>60</th>
<th>84</th>
<th>108</th>
<th>132</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative potency</td>
<td>112 ± 2.54</td>
<td>106 ± 2.87</td>
<td>104 ± 3.58</td>
<td>123 ± 5.64</td>
<td>116 ± 0.89</td>
<td>94 ± 3.67</td>
<td>109 ± 2.16</td>
<td>100</td>
</tr>
</tbody>
</table>

Values are mean ± SD of triple determinations

### Fig. 2: Mycelia dry weight accumulation (a), L-tyrosine (b) and L-dopa (c) concentrations in control culture (open square) and culture with proline added (0.05 g L\(^{-1}\)) at 60 h (filled square). All the data points represent mean values of three separate measurements.
Tyrosine effect on lincomycin production

**Influence of l-tyrosine on lincomycin production**

Since it was found that the addition of proline to the fermentation increased the biosynthesis of tyrosine, comprehension the effect of tyrosine on the regulation of lincomycin biosynthesis attracted our attention. In fact, the use of tyrosine in lincomycin production was done not just recently [23, 28], but they provided no information on the pathway involved and the producing strains used were wild-type strains rather than proline-resistant mutant.

In this study, two strains, the original strain (55-20) and the proline-resistant mutant (pro10–20) were compared for the effect of tyrosine on lincomycin production.

The basal culture medium was supplemented with different amounts of tyrosine (0.005, 0.01, 0.02, 0.05, 0.1, 0.2 and 0.5 g L\(^{-1}\)). The biosynthesis of lincomycin was limited for 55-20. The increasing initial additions of l-tyrosine decreased lincomycin productions, the more tyrosine was delivered to the medium, the less lincomycin were produced (Table 3). But for the mutant pro10–20, the behavior was different (Table 4). With increased addition of l-tyrosine, the curve of lincomycin production just like a “concave parabola”, that is, when the addition of tyrosine was relatively slight (at 0.005 and 0.01 g L\(^{-1}\)) or copious (at 0.1, 0.2 and 0.5 g L\(^{-1}\)), it favored lincomycin biosynthesis, while the tyrosine concentration was 0.02 or 0.05 g L\(^{-1}\) (the intermediate concentration), the production of lincomycin decreased.

To investigate the reason why lincomycin production decreased with the addition of tyrosine (at 0.02 and 0.05 g L\(^{-1}\)) by strain pro10–20, we determined the contents of l-tyrosine and l-dopa in the fermentation broth.

**Determination of the nature of l-tyrosine effect**

The medium was supplied with tyrosine at 0.005, 0.05, and 0.1 g L\(^{-1}\), respectively, and Fig. 3 shows the trends of tyrosine and dopa concentrations in the broth during the fermentation.

Three supplementations of tyrosine have slight stimulus on DMW, but for tyrosine and dopa, their concentrations were the highest when the added tyrosine amount was 0.05 g L\(^{-1}\), while the corresponding final concentration of lincomycin was the lowest. The high lincomycin titer were observed when 0.005 or 0.1 g L\(^{-3}\) of tyrosine was added (results not shown). It seemed that tyrosine and dopa were consumed less in the case of 0.05 g L\(^{-1}\) tyrosine addition than the other two additions (0.005 and 0.1 g L\(^{-1}\) of tyrosine) at the post-synthetic phase of lincomycin production (120–180 h). This may be connected with the biosynthesis of melanin and the activities of tyrosinase. It was reported [11] that the catalytic activities of tyrosinase could be adjusted by the concentration of l-dopa in the catalytic system. When the consumption and generation of dopa reach equilibrium, the rate to convert tyrosine to melanin will be maximized.

When traces of tyrosine (0.005 g L\(^{-1}\)) was added to the culture, the increase in dopa was limited, and the formed dopa was unable to meet the demand of propylproline, so nearly all the dopa were converted to propylproline. With a larger amount of tyrosine (0.05 g L\(^{-1}\)) addition, more dopa accumulated till the concentration of dopa reached equilibrium and the flux to melanin increased, resulting less propylproline formation and lower lincomycin production. At the same time, a huge amount of dopa and tyrosine left in the system. When 0.1 g L\(^{-1}\) of tyrosine was added, even more dopa accumulated and broke the balance then favored lincomycin synthesis again.

**Effect of l-tyrosine addition at different time of cultivation on lincomycin production**

In order to research the tyrosine effect on lincomycin production further, the influence of tyrosine feeding time on lincomycin biosynthesis was conducted. In consideration of production cost, we examined the optimal addition

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Relative lincomycin potency by the original strain (55-20) with increasing initial concentrations of l-tyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>l-tyrosine (g L(^{-1}))</td>
<td>0.005</td>
</tr>
<tr>
<td>Relative potency</td>
<td>97 ± 1.13</td>
</tr>
<tr>
<td>Values are mean ± SD of triple determinations</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Relative lincomycin potency by the proline-resistant mutant (pro10–20) with increasing initial concentrations of l-tyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>l-tyrosine (g L(^{-1}))</td>
<td>0.005</td>
</tr>
<tr>
<td>Relative potency</td>
<td>110 ± 4.99</td>
</tr>
<tr>
<td>Values are mean ± SD of triple determinations</td>
<td></td>
</tr>
</tbody>
</table>
time of tyrosine with the least tyrosine addition (0.005 g L\(^{-1}\)). The results (Table 5) showed the suitable time for adding tyrosine is 0–12 h after incubation. Lincomycin potency increased by 11%.

### Table 5 The effect on lincomycin potency by pro-10–20 with addition of 0.005 g L\(^{-1}\) tyrosine at different time

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0</th>
<th>12</th>
<th>36</th>
<th>60</th>
<th>84</th>
<th>108</th>
<th>132</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative potency</td>
<td>110 ± 3.84</td>
<td>106 ± 1.58</td>
<td>94 ± 3.54</td>
<td>97 ± 2.87</td>
<td>102 ± 4.65</td>
<td>95 ± 2.64</td>
<td>101 ± 3.33</td>
<td>100</td>
</tr>
</tbody>
</table>

Values are mean ± SD of triple determinations.

### Influence of L-alanine

During the synthesis of propylproline, glucose is converted via glycolysis and the hexosemonophosphate shunt to phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P), respectively, which are in turn converted via the shikimic acid pathway to tyrosine and then to dihydroxyphenylalanine (L-dopa) and at last to propylproline [7]. PEP is instable and converted to pyruvic acid catalyzed by pyruvate kinase (PK) quickly. L-alanine is an inhibitor of PK and thus can direct the flux from PEP to the shikimic acid pathway to synthesize more tyrosine.

The influence of alanine on lincomycin production was studied first by the increasing alanine concentrations. The data in Table 6 indicate that addition of 0.1 g L\(^{-1}\) of alanine improved lincomycin production by 11%. Then the effect of the time to add 0.1 g L\(^{-1}\) of alanine was examined (Table 7). It can be seen that addition of 0.1 g L\(^{-1}\) of alanine could improve lincomycin production by 13%.

### Mechanism of the effect of L-alanine addition

In order to understand the mechanism about the effect of alanine addition on lincomycin biosynthesis, we determined the mycelia dry weight accumulation, the...
concentrations of pyruvic acid, tyrosine, dopa and the PK activities in the fermentation conducted with and without addition of 0.1 g L\(^{-1}\) alanine (Fig. 4).

The above results suggest that alanine seemed to inhibit the PK activity over the whole duration of the fermentation, and the effect was more intense during the later phase of lincomycin production (Fig. 4b). Concurrently, the pyruvic acid concentration was lower when alanine was added compared with the control culture (Fig. 4c). Contrary to pyruvic acid, more tyrosine and dopa were observed to accumulate in the culture with alanine added (Fig. 4d, e). Similar to tyrosine, alanine has a slight but depressive effect on the growth of *Streptomyces lincolnensis* (Fig. 4a). In light of these results, we can say that alanine has an inhibitory effect on pyruvate kinase and permits more PEP to be converted via the shikimic acid pathway to tyrosine to conduce to the biosynthesis of propylproline.

**Discussion**

Biosynthesis of lincomycin was investigated extensively since the 1960s of the twentieth century including the chemical structure [13, 14], cultivation [1, 2, 4], biosynthesis [20, 22] and chemical modification [5, 12]. In the last few years, investigators paid more attention to the industrial fermentation [16] rather than the metabolic pathway of it. Before that, various chemicals were introduced to the fermentation to induce the formation of lincomycin-related antibiotics [8, 23, 27, 28] among which propylproline attracts our attention. In the biosynthesis of lincomycin, propylproline has been proposed to be derived from \(L\)-tyrosine. It has also been found that \(L\)-3, 4-dihydroxyphenylalanine (\(L\)-dopa) is probably an intermediate in the pathway for the rare amino acid propylproline [6, 7]. So to supply some amino acids with relationships to tyrosine and dopa to increase the formation of propylproline and to determine the concentration of propylproline indirectly from them were presumed to be an economic and effective way.

Based on the previous report that propylproline was found to increase production of lincomycin [8], a mutant strain pro10–20 resistant to feedback regulation of proline (an analog of propylproline) was screened by rational screening.

Respective addition of three amino acids (\(L\)-proline, \(L\)-tyrosine, \(L\)-alanine) to the culture medium stimulated lincomycin production (Tables 1, 4, 6). These phenomena were probably caused by increased accumulations of tyrosine and dopa which are important intermediates for propylproline synthesis [6, 7].

\(L\)-proline seemed to have dual effects in this study: to screen out the mutant pro10–20 as an analog of propylproline; to serve as a nutritive material or something else to stimulate tyrosine biosynthesis. This stimulation was increased when proline was delivered at the start of the antibiotic production phase (60 h) probably because sufficient tyrosine converted from added proline met the requirements for the biosynthesis of more propylproline.

Addition of tyrosine to the medium for culture of the original strain 55-20 confirmed the inhibitory effect of propylproline on lincomycin production. The added tyrosine increased accumulation of propylproline and the excess propylproline repressed biosynthesis of lincomycin. But for the mutant strain pro10–20, which has removed the inhibitory characteristic, the addition of tyrosine favored the biosynthesis of lincomycin. Furthermore, the addition of tyrosine showed a “critical phase” during which the added tyrosine directed some metabolic flux to melanin. So it is necessary to make an adjustment on the concentration of tyrosine added to get a productive and effective method to divert the metabolic flow to where we needed, that is to permit more tyrosine converted to propylproline.

The biosynthesis of tyrosine consists of shikimic acid pathway that shares the common intermediate phosphoenolpyruvate (PEP) from which pyruvic acid is synthesised.

<table>
<thead>
<tr>
<th>Table 6</th>
<th>Lincomycin production with different contents of (L)-alanine addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>(L)-alanine (g L(^{-1}))</td>
<td>0.05</td>
</tr>
<tr>
<td>Relative potency</td>
<td>106 ± 3.56</td>
</tr>
<tr>
<td>Values are mean ± SD of triple determinations</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 7</th>
<th>Effect of (L)-alanine (0.1 g L(^{-1})) addition at different times on lincomycin production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (h)</td>
<td>0</td>
</tr>
<tr>
<td>Relative potency</td>
<td>113 ± 2.69</td>
</tr>
<tr>
<td>Values are mean ± SD of triple determinations</td>
<td></td>
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</tbody>
</table>
Addition of alanine blocked in the synthesis of pyruvic acid by inhibiting the activity of pyruvate kinase and led to accumulation of the end product (tyrosine) of the other branch through redirecting of the common intermediate (PEP). This is in turn favorable to propylproline biosynthesis and increases lincomycin production. Based on the study of the preparation of proline-resistant mutant strain pro10–20 and fermentation improving by amino acids supplementation, we discussed the propylproline effect on lincomycin biosynthesis according to the metabolic pathway and the understanding of this effect could be useful for future biotechnological application.

**References**
