Effect of Lauryl Alcohol on Production of Taxanes in a Suspension Callus Culture

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(Received September 10, 2013; Accepted October 25, 2013)

We report here the effect of lauryl alcohol (LA), an effective organic solvent for in situ extraction for production of paclitaxel, on the production of taxanes (10-deacetyl baccatin III, baccatin III and cephalomannine) in a suspension callus culture of Taxus cuspidata. The culture conditions for enhancing production of the taxanes by avoiding feed-back inhibition of paclitaxel were examined. Increased callus growth and production amounts of the taxanes except cephalomannine were obtained in the LA-medium culture systems compared to the control culture where LA was not added. This result suggested that LA influenced the cellular and/or enzymatic activities involved in the biosynthetic pathway of paclitaxel.

1. Introduction

Paclitaxel (Taxol®) demonstrates unique anti-mitotic activity by promoting the assembly of tubulin and stabilizing the resulting microtubules [1]. It is an excellent antitumor drug against various types of cancers [2-4] and shows potential for treating HIV-associated Kaposi’s sarcoma [5] and Alzheimer’s disease [6]. Paclitaxel is semi-synthetically produced through many steps of reactions from precursors such as 10-deacetyl baccatin III or baccatin III which are extracted from intact needles of the Taxus yew tree. Extracting the precursors for the semisynthetic process leads to consumption of a large quantity of needles and is very expensive. Plant cell (callus) culture is a sustainable and promising process for production of paclitaxel because it is easily induced from a small piece of plant tissue such as a leaf and stem and propagated for a long time using a suitable culture medium under a controlled environment. However, callus growth is suppressed by feedback inhibition of paclitaxel produced in the callus cultures of the Taxus species [7]. To avoid the inhibition by paclitaxel, two phase culture systems using solid adsorbents [8] or water-immiscible organic solvents [9-11] have been employed for the selective in situ extraction from culture medium based on paclitaxel’s hydrophobicity. We found that higher alcohols and hydrocarbons with higher log P values as well as triglycerides were effective [7] and reported that lauryl alcohol (LA) in particular increased the paclitaxel productivity in the two phase culture systems [7, 12-13]. Addition of methyl jasmonate (MJ), which is known as an elicitor stimulating production of secondary metabolites such as paclitaxel, into the culture systems increases paclitaxel production [13-14]. In the callus culture 10-deacetyl baccatin III and baccatin III are intermediates in the biosynthetic pathway of paclitaxel (Figure 1), and cephalomannine is a byproduct in the paclitaxel production process. Though 10-deacetyl baccatin III, baccatin III and cephalomannine aren’t antitumor agents, 10-deacetyl baccatin III and baccatin III can be utilized as precursors for paclitaxel synthesis by the semisynthetic method. The cultured callus is able to
produce paclitaxel directly and the related taxanes such as 10-deacetyl baccatin III and baccatin III as sources for the semisynthetic process simultaneously. However, the production of these taxanes in the LA-medium two phase culture systems has never been reported.

Many researchers report that the amounts of paclitaxel and the related taxanes produced in the culture including MJ are greater than those in the control culture where MJ was not added. The data suggest that the production amounts of the related taxanes are dependent on the paclitaxel concentration. In the present research, the production of the related taxanes in a callus culture by in situ extraction with LA, where the feedback inhibition of paclitaxel was reduced, was examined.

2. Experimental

2.1 Callus and Medium

The callus induced from the leaves of a Taxus cuspidata tree planted in Sojo University was subcultured in a solid medium containing an agar (Gellan gum, Wako Pure Chemical Co., Osaka, Japan) at 25°C in the dark [7]. The medium for the callus culture was a modified Gamborg's B5 [16] medium where the potassium nitrate concentration was reduced to 10 mM and ammonium sulfate was eliminated [17].

2.2 Lauryl alcohol and the taxane partition coefficient

Lauryl alcohol (LA), a fatty alcohol, was used for the in situ extraction of paclitaxel and the related taxanes. [15]

<table>
<thead>
<tr>
<th>Taxane</th>
<th>Partition coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-Deacetyl baccatin III</td>
<td>2.6</td>
</tr>
<tr>
<td>Baccatin III</td>
<td>2.1</td>
</tr>
<tr>
<td>Cephalomannine</td>
<td>3.3</td>
</tr>
</tbody>
</table>
| Paclitaxel         | 2.3

a. Ref. 7

Table 1. Properties of lauryl alcohol (LA)

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular formula</td>
<td>( \mathrm{CH}_3(\mathrm{CH}<em>2)</em>{10}\mathrm{CH}_2\mathrm{OH} )</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>186.33 g/mol</td>
</tr>
<tr>
<td>Melting point</td>
<td>24 °C</td>
</tr>
<tr>
<td>( \log P )</td>
<td>5.0</td>
</tr>
</tbody>
</table>

\[ \text{DBBT}: \text{taxane } 2a-O\text{-benzoyltransferase} \]
\[ \text{DBAT}: 10\text{-deacetylbaccatin III-10-O-acetyltransferase} \]
\[ \text{BAPT}: \text{baccatin III: 3-amino, 3-phenylpropanoyltransferase} \]
\[ \text{DBTNBT}: 30\text{-N-debenzoyl-2-deoxytaxol-N-benzoyltransferase} \]
related taxanes from the culture medium in the present research. The properties of LA are shown in Table 1. LA having a higher log $P$ value is reported to be an effective solvent for the extraction of paclitaxel from the culture medium and to enhance the productivity of paclitaxel [7, 10-14]. LA has higher partition coefficients for the related taxanes (Table 1), resulting in easy extraction and recovery of these compounds from the culture medium.

2.3 Callus culture

Suspension callus cultures were performed in a LA-medium two phase system as previously reported [14]. Briefly, the suspension culture inoculated by the precultured callus was carried out in a 100 mL Erlenmeyer flask containing 20 mL of the medium with 10% of LA on a rotary shaker (NR-150, Taiotec, Saitama, Japan) at 110 rpm in the dark at 25 °C. After a 14 d culture period, a sample was withdrawn from the culture. The amount of fresh cells was measured. The concentrations of paclitaxel and the related taxanes in the medium phase, the LA phase and the callus were analyzed in all samples.

To investigate the effectiveness of LA on production of the taxanes, the following two culture conditions were examined. All the cultures under the same conditions were performed in triplicate.
1. Callus culture with in situ extraction with LA (This culture was designated “LA”.)
2. Callus culture in the absence of LA (This culture was conducted as the control and designated "control").

2.4 Analysis

Extraction of paclitaxel and the related taxanes was conducted according to the procedure reported previously [14]. The extracted paclitaxel and taxanes were separated by using a reversed-phase high performance liquid chromatograph system equipped with a silica-based column (Luna PFP (2), Phenomenex, USA) and detected by UV absorbance at 225 nm according to the manufacture’s protocol. The fresh cell weight (FCW) after harvesting, washing and drying on filter paper under vacuum was determined by weighing.

3. Results and Discussion

After initializing suspension callus culture, it was observed that taxanes were produced and a part of them partitioned in LA. Paclitaxel, 10-deacetyl baccatin III, baccatin III and cephalomannine were analyzed by reversed-phase HPLC. The fresh cell weight (FCW) and concentration of the taxane in the medium after the 14 d culture period in the LA-medium two phase systems are shown in Figures 2 and 3, respectively. The FCW in the LA-medium two phase systems was 1.1-fold higher than that in the control culture. The paclitaxel concentration in the medium in the culture including LA was 0.018 mg/L, while that in the control was 0.032 mg/L (Figure 3). The paclitaxel concentration in the LA was 0.042 mg/L which was a similar value to that calculated theoretically from the partition coefficient of paclitaxel in Table 1. The decreased paclitaxel concentration in the medium including LA was due to the partitioning of paclitaxel into the LA phase. The paclitaxel concentration inhibiting the callus growth of *T. cuspidata* was estimated to be higher than 0.02 mg/L (data not shown). Therefore, the higher callus growth in the culture including LA resulted from the decreased paclitaxel concentration in the medium of less than 0.02 mg/L by in situ extraction of paclitaxel with LA. The improved callus growth rates in a LA-medium two phase systems including various volume fractions of LA where the paclitaxel concentration in the medium was less than 0.02 mg/L was observed [7].
The concentrations of paclitaxel and cephalomannine in the medium with LA decreased compared to those in the control. In contrast, the concentrations of 10-deacetyl baccatin III and baccatin III in the medium with LA increased compared to those in the control. These results might be due to the cellular and/or enzymatic activity related in its metabolism (Figure 1) by addition of LA.

The total production amount of paclitaxel, which is the sum of paclitaxel in the medium, LA and the callus, in the LA-medium two phase culture systems was slightly higher than that in the control culture (Figure 4). This is because of the reduction of the feed-back inhibition by paclitaxel as described above. It is also supposed that the produced paclitaxel was converted to other compounds in the culture including LA. The total production amounts of 10-deacetyl baccatin III and baccatin III in the culture including LA were also greater than that in the control. The greater production of the taxanes except cephalomannine in the culture with LA could be explained by the reduced paclitaxel’s feed-back inhibition which contributed to improving cellular and/or enzymatic activity, and consequently increased the biosynthesis of these taxanes. On the other hand, a positive correlation between the transcript abundance of genes involved in the biosynthesis of paclitaxel and the amount of taxane production has been reported [18-20]. The expression of genes encoding the enzymes in the taxane synthesis in the callus culture with in situ extraction with LA is unknown. The amount of cephalomannine production in the presence of LA was reduced compared to that in the control (Figure 4). In order to estimate the biosynthetic efficiency of paclitaxel, the paclitaxel synthetic ratio, which

![Figure 2. Callus growth after the 14 d culture period under each culture condition. Each error bar shows a standard error calculated from three independent experiments.](image2)

![Figure 3. Concentration of taxane in the medium after the 14 d culture period under each culture condition. Each error bar shows a standard error calculated from three independent experiments. Dotted line shows the inhibition concentration of paclitaxel.](image3)

![Figure 4. Total amount of taxane after the 14 d culture period under each culture condition. Each error bar shows a standard error calculated from three independent experiments.](image4)
is defined as the production amount of paclitaxel to that of cephalomannine, was introduced. The paclitaxel synthetic ratios in the culture including LA and in the control are calculated to be 0.48 and 0.22 (Figure 4), respectively, suggesting that \textit{in situ} extraction with LA effectively suppresses the production of cephalomannine and/or promotes synthesis of paclitaxel rather than cephalomannine (Figure 4) though scattering in amounts of the produced cephalomannine were observed. It is considered that the increased production of paclitaxel due to \textit{in situ} extraction with LA conversely reduced the production of cephalomannine because cephalomannine is a byproduct in the biosynthetic pathway of paclitaxel (Figure 1). The expression profile of genes (Figure 1) in the \textit{Taxus cuspidata} cell line P1991 elicited by methyl jasmonate (MJ) was examined by Mims \textit{et al.}[18]. In the MJ-elicited culture, mRNA levels of \textit{DBBT} and \textit{DBAT} increased, however, those of \textit{BAPT} and \textit{DBTNBT} did not increase. A similar gene expression might be occurring in the present culture including LA because higher concentrations of the taxanes in the LA-medium two phase systems were obtained (Figure 4). This explanation is not obvious and thus should be further investigated taking genes expression encoding the enzymes involved in the biosynthetic pathway of the taxanes into account. However, the production amount of each taxane in the callus culture might be subjected to the characteristics of \textit{T. cuspidata}, because the contents of the taxanes depend on the plant species [21].

It is clearly shown that the present callus culture by \textit{in situ} extraction with LA, where the feedback inhibition of paclitaxel was reduced, was effective in increasing the production of 10-deacetyl baccatin III, baccatin III and paclitaxel. However, the productivity of each taxane in the LA-medium two phase culture systems is somewhat smaller comparing to the results obtained by other researchers. The productivities of the paclitaxel and baccatin III in the present culture including LA are calculated to be 0.023 and 0.014 mg/(L d), respectively, while values of 0.029 and 0.029 mg/(L d) in a suspension culture of \textit{T. baccata} have been reported by Yukimune \textit{et al.} [22]. In order to enhance the productivities of the taxanes the following attempts should be considered for further examination. Increasing the volume fraction of LA, which improved the callus growth and the paclitaxel production [11], might contribute to enhancement of the taxane synthesis. Addition of an elicitor, methyl jasmonate (MJ), into the medium leads to an increase in the production of paclitaxel and baccatin III by a factor of one hundred compared to the control culture [22]. The LA-medium two phase culture system including MJ, which enhanced the production of paclitaxel [13-14], might give an increase in taxane production. In addition, sequential refreshment of LA including MJ [14] must be suitable for this purpose in the present culture systems. We also reported that water-soluble 5-aminolevulinic acid (ALA), which promoted the growth and yield of the plant [23-25], had positive effects on the callus growth and biosynthesis of paclitaxel [26]. The culture conditions in the LA-medium two phase culture systems including ALA and MJ for efficient callus growth and higher taxane production are under investigation.

4. Conclusion

The \textit{in situ} extraction effect of lauryl alcohol (LA) on the callus growth and taxane production in the suspension culture of \textit{T. cuspidata} callus was investigated. The culture in the LA-medium two phase systems improved the callus growth due to the decrease of the paclitaxel concentration in the medium, \textit{i.e.}, decreased the feed-back inhibition of paclitaxel, and increased the production of 10-deacetyl baccatin III,
baccatin III and paclitaxel except cephalomannine. It was observed that the present culture system by \textit{in situ} extraction with LA was effective to promote synthesis of paclitaxel rather than cephalomannine. Since the productivities of these taxanes in the present research were somewhat smaller than those reported by other researchers, culture conditions such as a higher volume fraction of LA and/or an elicitor addition are now under investigation to enhance the production of these taxanes.

\textbf{References}

