Enhanced Productivity of Paclitaxel and Related Taxanes in Plant Cell Culture Including Aliphatic Ionic Liquids

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We report here on hydrophobic ionic liquids (ILs) which enhance the productivity of paclitaxel and the related taxanes of 10-deacetyl baccatin III, baccatin III and cepharomannine with in situ extraction from an aqueous medium in the suspension cell culture of Taxus cuspidata in the IL-medium two phase culture system. Two aliphatic ILs of N-methyl-n-propylpiperidinium bis(trifluoromethane-sulfonyl)imide (PP13-TFSI) and N-methyl-n-butylpyrrolidinium bis(trifluoromethanesulfonyl)imide (P14-TFSI) were used. It was found that the two ILs had no cytotoxicity and enhanced productivity of paclitaxel in the cell culture. In particular, the more hydrophobic P14-TFSI, which enhanced the productivity of paclitaxel and the total taxanes by a factor of more than 2 compared to PP13-TFSI, could be an effective extractant.

1. Introduction

Paclitaxel (PX) is an expensive anticancer drug because the conventional semi-synthetic method requires many reaction steps of precursors such as baccatin III or 10-deacetyl baccatin III extracted from yew tree needles. A culture using callus induced from the needles is one of the promising methods for the cost-effective production of paclitaxel. However, there is a problem of feedback-inhibition of the paclitaxel produced in the culture. In order to reduce the paclitaxel’s inhibition, two phase culture systems using water-immiscible organic solvents, have been proposed for the in situ extraction of hydrophobic paclitaxel from the culture medium [1-2]. We investigated the use of hydrophobic ionic liquids (ILs) for the in situ extraction of paclitaxel from the aqueous medium [3] and reported that 1-hexyl-3-methylimidazolium hexafluorophosphate (HMIN-PF₆) extracted paclitaxel from the aqueous medium [4]. ILs have gained considerable attention as safer solvents in contrast to conventional organic solvents because of their unique properties such as negligible volatility, high thermal stability, and selective solubility. ILs have been applied for extraction and separation of bioactive compounds [5-9]. It is reported that HMIN-PF₆ extracted hydrophobic 3-indole-butyric acid from pea plants [10]. Ferulic acid and caffeine acid found in various plants could be readily extracted with HMIN-PF₆ [11]. Recently, higher extraction efficiencies were obtained for DNA molecules using magnetic ILs such as benzylltrioctylammonium bromotrichloroferrate (III) ([(C₈)₃BnN⁺][FeCl₃Br⁻]) and 1,12-di(3-hexadecylbenzimidazolium) dodecane bis[(trifluoromethyl)sulfonyl]imide bromotrichloroferrate (III) ([(C₁₆BnIM)₂C₁₂²⁺][NTf₂⁻, FeCl₃Br⁻]) [12]. Though there is a report on enhancement of extraction efficiency of paclitaxel from biomass taken from the culture broth.
using a co-solvent made of 1-butyl-3-methylimidazolium tetrafluorophosphate (BMIN-BF$_4$) and methanol under acidic conditions [13], there is no report on the in situ extraction of paclitaxel and the related taxanes of 10-deacetyl baccatin III (10-DAB), baccatin III (BIII) and cepharomannine (CM) in the plant cell culture with the ILs except in our study [4]. Figure 1 shows the main metabolic pathway of PX and the related taxanes from geranylgeranyl diphosphate [14].

Recently, novel hydrophobic ILs such as aliphatic $N$-methyl-$n$-propylpiperidinium bis(trifluoromethanesulfonyl)imide (PP13-TFSI) [15] and $N$-methyl-$n$-butylpyrroloidinium bis(trifluoromethanesulfonyl)imide (P14-TFSI) have been produced. In the present research the effect of PP13-TFSI and P14-TFSI on the productivity enhancement of PX and the related taxanes of 10-DAB, BIII and CM by the in situ extraction in plant cell culture were investigated considering their cytotoxicity. This is a first report on the application of the ILs for in situ extraction of the taxanes from the plant cell culture.

2. Experimental

2.1 Reagents

Two aliphatic ILs of $N$-methyl-$n$-propylpiperidinium bis(trifluoromethanesulfonyl)imide (PP13-TFSI) and $N$-methyl-$n$-butylpyrroloidinium bis(trifluoromethanesulfonyl)imide (P14-TFSI) purchased from Kanto Chemical Co. (Tokyo, Japan) were used. Their physical properties and chemical formulas are shown in Table 1. The partition coefficient of PX in the IL-medium two phase system was measured according to the procedure described elsewhere [3]. The solubility of the IL in the aqueous medium was measured by the following method. A mixture of 50 µL IL ($X_0$ [mg]) and 1 mL of the medium in a microtube was vigorously shaken and stood overnight at 26 °C. After complete removal of the medium phase in the microtube, the residual amount of IL ($X_1$ [mg]) was measured. The solubility of IL in the medium was estimated by Equation (1).

\[
\text{Solubility of the IL in the medium [mM]} = \frac{1000 \times (X_0 - X_1)}{\text{molecular weight of IL}} \quad (1)
\]

1-Hexyl-3-methylimidazolium hexafluorophosphate (HMIN-PF$_6$, TCI, Tokyo, Japan), which was found to enhance the productivity of the taxanes in the previous report [14], was used to compare the efficiency of the taxane production with the two ILs.
2.2 Plant cell culture

Callus induced from the needles of *Taxus cuspidata* and a modified Gamborg’s B5 medium [1] were used for the culture. Suspension culture inoculated by the precultured cells was carried out in a 100 cm³ Erlenmeyer flask containing 20 cm³ of the B5 medium and 1 cm³ of the ILs (5 vol%) on a rotary shaker (NR-150, Tai-tec, Saitama, Japan) at 110 rpm in the dark at 26 °C. During the culture the amounts of fresh cells, paclitaxel and the related taxanes in the culture flask were measured.

2.3 Evaluation of cytotoxicity and effectiveness for taxane production of ILs

To examine the cytotoxicity and the effectiveness for the taxanes production of ILs, the suspension cell culture in the 100 mL Erlenmeyer flask including 0.4 g fresh cells and 20 mL medium by *in situ* extraction with 5 vol% IL was carried out. The suspension cell culture in the absence of the IL was conducted as the control culture.

For examination of the cytotoxicity, the relative cell growth rate, $R_{FCW}$, was defined as follows,

$$R_{FCW} = \frac{FCW_{IL}}{FCW_C}$$

where $FCW_{IL}$ and $FCW_C$ are the fresh cell weights in the cultures including the IL and that in the control after a 7 day culture period, respectively.

If an IL has cytotoxicity against the cells, the value of $R_{FCW}$ value will be less than 1.

For evaluation of the effectiveness of the IL on the productivity of the taxanes, the specific production rate of the taxane, $E_{taxane}$ was defined as follows,

$$E_{taxane} [\mu g/(g-cell \cdot d)] = \frac{\Delta P (x \cdot \Delta t)}{x \cdot \Delta t}$$

where $\Delta P$ is the amount of taxane (10-DAB, BIII, PX, CM and the total amount of these taxanes (total)) produced during a culture period of $\Delta t$, $\Delta t$ is 7 d and $x$ is the fresh cell weight after a culture period of $\Delta t$.

<table>
<thead>
<tr>
<th>IL</th>
<th>Chemical formula</th>
<th>Abbreviation</th>
<th>Solubility in aqueous medium [mM]</th>
<th>Partition coefficient of paclitaxel in the IL-medium two-phase system [-]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Hexyl-3-methylimidazolium hexafluorophosphate</td>
<td>HMIN-PF₆</td>
<td></td>
<td>29.5</td>
<td>160</td>
</tr>
<tr>
<td>N-Methyl-n-propylpiperidinium bis(trifluoromethanesulfonylimide)</td>
<td>PP13-TFSI</td>
<td></td>
<td>19.9</td>
<td>37.1</td>
</tr>
<tr>
<td>N-Methyl-n-butylpyrroloidinium bis (trifluoromethanesulfonylimide)</td>
<td>P14-TFSI</td>
<td></td>
<td>16.1</td>
<td>45.4</td>
</tr>
</tbody>
</table>

2.4 Analysis

Cells were harvested from cultures, washed with water, blotted on filter paper to remove excess liquid, and then weighed to determine the FCW. The amounts of the paclitaxel and the related taxanes in the medium phase, the IL phase and the cells in all samples were analyzed by using a reversed-phase HPLC...
3. Results and Discussion

3.1 Cytotoxicity of ILs

The weight of the fresh cells in the culture including each IL after a 7 day culture period is shown in Figure 2. Regardless of the types of the ILs, the fresh cells weight in the culture with the IL was similar to that in the control culture and the values of $R_{FCW}$ under all the culture conditions were almost 1, indicating that all the ILs used in the present research have no cytotoxicity.

3.2 Effects of ILs on the productivities of paclitaxel and the related taxanes

Figure 3 shows the effect of the ILs on the productivity of PX, $E_{PX}$, which is defined by Equation (2). The values of $E_{PX}$ in the cultures including the ILs increased compared to that in the control culture. This increase might be caused by partition of the produced PX into the ILs. The $E_{PX}$ value of P14-TFSI was 2 times higher than that of PP13-TFSI. P14-TFSI was more effective in the extraction and production of PX than PP13-TFSI and HMIN-PF$_6$ (Figure 3), because of its stronger hydrophobicity than PP13-TFSI due to its lower solubility in the aqueous medium than that of PP13-TFSI (Table 1).

The ILs might affect the activities of enzymes related to the biosynthesis of PX. Thus, the productivities of the related taxanes of 10-DAB, BIII and CM (Figure 1) in the culture including the ILs were examined. As shown in Figure 3, the productivities of the taxanes in the culture with the ILs were greater than those of the control culture. Greatest productivities of the total taxanes, which means the total sum of the taxanes, in the culture with P14-TFSI, were observed. The value of $E_{total}$ in the culture with P14-TFSI was 2 times greater than that with PP13-TFSI. These results suggest that P14-TFSI is an excellent extractant of PX and a stimulator for the enzymes related to the metabolic
pathway of paclitaxel synthesis in the plant cell culture. Production of secondary metabolites such as the taxanes is enhanced by the defense response to biotic or abiotic stresses. An enhancement of paclitaxel production by *T. cuspidata* cells was observed in an oleic acid-medium two phase system where the oleic acid provides the abiotic stress [16]. The ILs used in the present research might provide an abiotic stress like an elicitor to the plant cells, resulting in the greater productivities of the taxanes. As the reason for the stimulation of the metabolism was still unclear, this should be further studied. The reason why the greater productivity of the taxanes in the culture including PP13-TFSI or P14-TFSI compared to that including HMIN-PF₆ was obtained is also not clear. The higher permeation of PP13-TFSI or P14-TFSI compared to HMIN-PF₆ into the cell membrane due to their lowered solubility in the medium (Table 1) might lead to the greater productivity of the taxanes. An effective method for the back-extraction of the target taxanes from the ILs should be explored. The back-extraction and recovery of the taxanes from the ILs by adjustment of pH was under consideration because adjustment of the medium pH contributed to the efficient back-extraction of hydrophobic ferulic acid and caffeine acid from HMIN-PF₆ [11].

4. Conclusion

For effective *in situ* extraction of the anticancer drug, paclitaxel, from the aqueous medium in the suspension cell culture of *T. cuspidata*, two aliphatic ILs of N-methyl-n-propyl-piperidinium bis(trifluoromethanesulfonyl)imide (PP13-TFSI) and N-methyl-n-butylpyrrolidinium bis(trifluoromethanesulfonyl)imide (P14-TFSI) as extractants were used in the cell culture. The two ILs were found to have no cytotoxicity and increased the productivities of paclitaxel and the related taxanes of 10-DAB, BIII and CM. Greatest productivities of paclitaxel and the total taxanes in the culture with P14-TFSI were observed, suggesting that P14-TFSI is an excellent extractant and stimulator for the enzymes related to the metabolic pathway of paclitaxel synthesis.

Acknowledgement

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References