Production of Taxanes in an Ionic Liquid-medium Two Phase Culture System

Shinjiro YAMAMOTO*, Ryo NOGUCHI, Azumi HAYASHI, Yumiko KURIYAMA, Shuhei HAYASHI and Hitoshi MIYASAKA
Department of Applied Life Science, Sojo University, 4-22-1 Ikeda, Kumamoto 860-0082, Japan
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We report here the effect of a hydrophobic ionic liquid (IL), 1-hexyl-3-methylimidazolium hexafluorophosphate ([C₆MIM][PF₆]), for in situ extraction of hydrophobic taxanes such as 10-deacetyl baccatin III, baccatin III, cepharomannine and paclitaxel from an aqueous medium, on the production of the taxanes by Taxus cuspidata callus in the IL-medium two phase culture system. The effect of an additional elicitor, methyl jasmonate (MJ), on the production of the taxanes was also examined. The callus growth in the culture with the IL decreased compared to that without the IL. The production amount of 10-deacetyl baccatin III in the culture with the IL was enhanced significantly, which might cause the callus growth to decrease. Addition of MJ in the culture with the IL promoted the amount of cepharomannine produced. It was clear that [C₆MIM][PF₆] was an effective solvent for extraction and production of the taxanes in the callus culture.

1. Introduction

Paclitaxel, one of the taxanes, is an anti-cancer drug [1-3] and could be a candidate as a therapeutic agent against Alzheimer's disease [4]. Manufactured paclitaxel is semi-synthetically produced from precursors such as baccatin III or 10-deacetyl baccatin III extracted from yew tree needles. Since the synthesis of the precursors to paclitaxel requires many steps of reactions and isolation, paclitaxel is very expensive. The steps include the utilization of some harmful organic solvents [5], meaning that the semi-synthetic method is a non-sustainable process for making paclitaxel. Plant cell culture is a sustainable and reliable method for safe and effective production of paclitaxel. However, there is a problem because cell growth is inhibited by the paclitaxel produced in the cultures [6]. In order to decrease the paclitaxel’s inhibition, two phase culture systems using solid adsorbents [7] or water-immiscible organic solvents [8-11] have been proposed for the in situ extraction of hydrophobic paclitaxel from the culture medium. We reported that lauryl alcohol (LA) is an effective organic solvent for this purpose, while the partition coefficient of the taxanes into the LA-medium two phase systems at 26 °C is 2.3 [12]. Thus, we investigated the use of hydrophobic ionic liquids (ILs) for the in situ extraction of paclitaxel from the aqueous medium [13]. ILs have attracted considerable attention as safer solvents in contrast to conventional organic solvents because of their unique properties such as negligible volatility, non-flammability, high thermal stability, and selective solubility. ILs have been applied for extraction and separation of bioactive compounds (14-18). It is reported that 1-hexyl-3-methylimidazolium hexafluorophosphate ([C₆MIM][PF₆]) extracted more hydrophobic 3-indole-butyric acid (IBA) from pea plants than 1-butyl-3-methylimidazolium
hexafluorophosphate ([C₄MIM][PF₆]) and an increase in the chain length of 1-alkyl group of imidazolium ionic liquid, [C₄MIM][PF₆]), increased the extraction efficiency of IBA [19]. Ferulic acid and caffeine acid found in various plants could be more readily extracted with [C₆MIM][PF₆] compared to ([C₄MIM][PF₆]) [20]. However, there is no report on the in situ extraction of the taxanes by the ILs in the plant cell culture.

In the present research, [C₆MIM][PF₆], selected for its non-cytotoxicity and higher partition coefficients of the taxanes [13], was investigated for the effective in situ extraction and production of taxanes such as 10-deacetyl baccatin III (10-DAB), baccatin III (BIII), cepharomannine (CM) and paclitaxel (PX) from an aqueous medium in the culture of Taxus cuspidata callus. The compounds of 10-DAB and BIII are intermediates and CM is a byproduct in the metabolic pathway of PX (Figure 1). This first report examines the effects of the IL on the callus growth and production of the taxanes. In addition, the effect of methyl jasmonate (MJ, Figure 2), an elicitor stimulating production of secondary metabolites such as paclitaxel in plant cells [12, 21], on the IL–medium two phase culture systems was examined.

2. Experimental

2.1 Callus, medium and reagents

Callus induced from Taxus cuspidata leaves was subcultured in a solid medium containing agar at 26 °C in the dark [6] and used in the present research. A modified Gamborg's B5 medium [22], where the potassium nitrate concentration was reduced to 10 mM and ammonium sulfate was removed [23], was used for the callus culture.

The taxanes, 10-DAB, BIII, CM and PX purchased from Wako Pure Chem. Co. (Osaka, Japan), were used as standard reagents for HPLC analysis.
1-Hexyl-3-methylimidazolium hexafluorophosphate ([C₆MIM][PF₆]), purchased from Tokyo Chemical Industry Co. (Tokyo, Japan), was selected because of the higher partition coefficients of the taxanes and its non-cytotoxicity, and used in the present research. The properties of [C₆MIM][PF₆] are shown in Table 1. The hydrophobic [C₆MIM][PF₆] has greater partition coefficients for the taxanes in [C₆MIM][PF₆]-medium two phase systems (Table 1), indicating that it is an excellent extractant for the recovery of the taxanes from the medium in the callus culture.

2.2 Callus culture in the IL-medium two phase systems

Suspension culture inoculated by the precultured callus was carried out in a 100 cm³ Erlenmeyer flask containing 20 cm³ of the aqueous medium and 1 cm³ of [C₆MIM][PF₆] (5 vol%) on a rotary shaker (NR-150, Taitec, Saitama, Japan) at 110 rpm in the dark at 26 °C. After a 14 d culture period, the amount of fresh cells in the culture flask was measured. The concentrations of the taxanes in the medium phase, the IL phase and the callus were also analyzed in all samples.

To investigate the effectiveness of the IL on the production of the taxanes, the following three culture conditions were examined.

1. Callus culture in the absence of IL: This culture was conducted as the control and designated "control".
2. Callus culture with in situ extraction with 5 vol% IL: This culture was designated “IL”.
3. Callus culture with in situ extraction with 5 vol% IL and elicitation with 10 mM MJ: This culture was designated “IL+MJ”.

For examining the effectiveness of the IL and MJ, the enhancement factor of callus growth, $E_{FCW}$, and that of the productivity of each taxane, $E_P$, were defined as follows,

$$E_{FCW} [-] = \frac{FCW_{IL}}{FCW_C}$$
$$E_P [-] = \frac{P_{IL}}{P_C}$$

where $FCW_C$, $FCW_{IL}$, $P_C$ and $P_{IL}$ are the fresh cell weight in the control, the fresh cell weight in the “IL” or “IL+MJ” cultures, the amount of each taxane in the control and the amount of each taxane in the “IL” or “IL+MJ” cultures after the 14 d culture periods.

2.3 Analysis

For measurement of the contents of the taxanes in the cells, the IL and the medium, each sample was prepared as follows. The samples in the cells and the medium were prepared in methanol according to the procedure reported previously [12]. The sample of the IL phase was collected in another tube and mixed with methanol (1:20, v/v). All the samples solubilized in methanol were separated by using a reversed-phase HPLC system (Shimadzu, Tokyo) 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.

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### Table 1. Properties of [C₆MIM][PF₆] (25 °C).

| Property               | Value       |
|------------------------|-------------|
| Molar mass             | 312.21 g/mol|
| Density                | 1.304 g/cm³ a|
| Viscosity              | 800 mPa·s a |
| Partition coefficients of taxanes in [C₆MIM][PF₆]-medium two phase systems | | |
| 10-Deacetyl baccatin III | 1100       |
| Baccatin III           | 60          |
| Cephalomannine         | 300         |
| Paclitaxel             | 160         |

a. Ref. 18
3. Results and Discussion

Figure 3 shows the effect of the IL on the callus growth in the culture. A decrease of callus growth in the “IL” culture was observed compared to that in the “control” culture regardless of the non-cytotoxicity of the IL. The reason for the decreased callus growth is probably due to the excess production of the metabolites as described below.

The total production amount of the taxanes, which is the sum of the taxanes in the cells, the medium and the IL, produced in the cultures with in situ extraction by the IL is shown in Figure 4. In the “control” culture, 2.5 µg/cm$^3$ 10-DAB, 0.43 µg/cm$^3$ BIII, 2.4 µg/cm$^3$ CM and 0.59 µg/cm$^3$ PX after the 14 d culture periods, which were commonly observed in the suspension callus culture, were obtained. The amounts of the taxanes except 10-DAB in the “IL” culture decreased compared to that in the “control” culture. BIII was not detected by reversed-HPLC analysis in the “IL” culture. The production amount of 10-DAB in the “IL” culture was 900 µg/cm$^3$, which was estimated to be more than three hundred times that in the “control” culture. The observed decrease in the callus growth in the “IL” culture as shown in Figure 4 is probably due to the significant accumulation of 10-DAB in the culture. It is also assumed that the activity of DBAT, which catalyzes the reaction of 10-DAB to BIII (Figure 1), was inhibited or decreased by the IL. The smaller amount of CM and the small amount of PX production observed in the “IL” culture, resulted from the decreased metabolism after 10-DAB by the IL.

The addition of MJ, which promotes the productivity of the secondary metabolites such as the taxanes, into the “IL” culture did not enhance the production amount of 10-DAB but increased the production of BIII and CM, because MJ promoted the metabolism after 10-DAB. Yukimune et al. have reported that BIII and PX production was significantly increased by elicitation with MJ compared to the control [24]. However, little production of paclitaxel even in the “IL+MJ” cultures was observed. It is supposed that the metabolic pathway in the “IL+MJ” culture preferred CM to PX in the present culture conditions.
Since the reason of the decreased metabolism after the production of 10-DAB in the “IL” culture was not clear, this should be further studied. Appropriate methods for a metabolic pathway to synthesize PX rather than CM should be examined in future. More hydrophobic ILs such as aliphatic amine-based ionic liquids are worthy of examination for this purpose. An effective process for the back-extraction of target taxanes from the IL should be developed. Since a lower partition coefficient of PX in organic solvent (tricaprylin)-medium two phase systems was obtained by reducing the medium pH [25], adjustment of the medium pH probably contributes to the efficient back-extraction and recovery of the taxanes from the IL.

4. Conclusion

For effective in situ extraction of the hydrophobic taxanes 10-deacety baccatin III, baccatin III, cepharomannine and paclitaxel from the aqueous medium in the culture of T. cuspidata callus, hydrophobic 1-hexyl-3-methylimidazolium hexafluorophosphate ([C\textsubscript{6}MIM][PF\textsubscript{6}]) as an ionic liquid was selected and used in the callus culture. [C\textsubscript{6}MIM][PF\textsubscript{6}] decreased the callus growth, while it increased the production amount of 10-DAB significantly. The decreased callus growth is probably due to the excess accumulation of 10-DAB. This accumulation of 10-DAB probably results from the decreased or inhibited activity of the enzyme, DBAT, catalyzing the reaction of 10-DAB to BIII by the IL might cause the excess accumulation of 10-DAB. Addition of MJ into the “IL” culture was performed for enhancement of the taxanes. MJ promoted the metabolic rate of the conversion of 10-DAB to BIII and CM. The back-extraction of the taxanes from the IL should be further examined for an effective recovery process for the target taxanes.

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