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RAPID COMMUNICATION

Effect of pH on fractional precipitation for pre-purification of paclitaxel from plant cell cultures

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Abstract−Fractional precipitation is a simple, efficient method for pre-purifying paclitaxel from plant cell cultures of *Taxus chinensis*. The pH, a key process variable in fractional precipitation, was optimized in terms of the yield and purity of paclitaxel with a fixed methanol concentration in water (61.5%, v/v), paclitaxel content in the crude extract (0.5%, w/v), and storage time (14 hr). The maximum yield (~99%) and purity (~84.4%) of paclitaxel were obtained at pH 8.0 with potassium phosphate buffer. The precipitate from fractional precipitation was an amorphous state paclitaxel by analysis of XRD and SEM. Also, the particle size distribution of dried precipitate was a range of approximately 119-411 μm. The use of fractional precipitation in the pre-purification process allowed for rapid and efficient separation of paclitaxel from interfering compounds, and dramatically increased the yield and purity of the crude paclitaxel for subsequent purification steps.

Key words: Paclitaxel, Fractional Precipitation, pH, Optimization, Pre-purification

INTRODUCTION

Paclitaxel, discovered in 1971 from the bark of *Taxus brevifolia*, has been one of the most important anticancer agents in the past few decades. It is a chemotherapeutic agent used to treat various cancers, most commonly ovarian cancer, breast cancer, and Kaposi's sarcoma. It is also efficacious in the treatment of other cancer, including those of the colon and lung, as well as melanoma and lymphoma [1,2]. Paclitaxel production in plant cell cultures has the potential to provide sufficient quantities of paclitaxel. Cell culture offers the potential advantage of availability and reliable production by using a renewable resource, whereas bark stripping methods lead to the destruction of scarce plant material. In addition, chemical synthesis of paclitaxel is very complex and has a very low yield. A plant cell culture process is also more adaptable for increases in demand for paclitaxel [3,4].

Little information exists with regard to procedures which are directly scalable to commercial operations for isolation and purification of paclitaxel. Also, all of the previous purification methods primarily aim to obtain crude paclitaxel of low purity by employing solvent extraction and chromatography work, and they essentially provide paclitaxel-related compounds such as terpenoids, lipids, chlorophyll, and phenols, accompanying the paclitaxel of interest [5-9]. Accordingly, high purity of paclitaxel has not been obtained. Even with the use of chromatographic columns and large volumes of solvent during purification steps, a heavy load of impurities occurs on the columns. Since the solubility of paclitaxel in organic solvent is very low, its purity and yield during chromatography are not easily controlled, and crystallization steps are essential to obtain high-purity paclitaxel. Accordingly, the existing purification methods are impractical and there remains a need for a method of isolating high-purity paclitaxel in a simple and economical manner.

In our previous paper [10], we developed the fractional precipitation process for purification of paclitaxel from plant cell cultures. Fractional precipitation is a rapid, simple, and efficient procedure for the pre-purification of paclitaxel with high purity and yield. In this study, the pH, a key process variable in fractional precipitation, was optimized to obtain the high purity and yield of paclitaxel in pre-purification step. Also, the morphology and particle size of precipitate were analyzed by XRD and SEM. This pre-purification process minimizes solvent usage, and the scale and complexity of the high performance liquid chromatography (HPLC) equipment needed for paclitaxel purification.

MATERIALS AND METHODS

1. Plant Materials and Culture Conditions

The suspension cells originating from *Taxus chinensis* were maintained in darkness at 24 °C with shaking at 150 rpm. The cells were cultured in modified Gamborg's B5 medium [11] supplemented with 30 g/L sucrose, 10 mM naphthalene acetic acid (NAA), 0.2 mM 6-benzylaminopurine (BA), 1 g/L casein hydrolysate, and 1 g/L 2-(N-morpholino)ethanesulphonic acid (MES). Cell cultures were transferred to fresh medium every 2 weeks. In prolonged culture for production, maltose, 1 and 2% (w/v), was added to the culture medium at days 7 and 21, respectively, and 4 mM $AgNO_3$ was added on the initiation of culture as an elicitor [12]. Following culture, the biomass was recovered by using a decanter (Westfalia, CA150 Clarifying Decanter) and high-speed centrifuge (Alfa-Laval, BTPX 205GD-35CDEFP).

2. Analysis of Paclitaxel

An HPLC system (Waters, USA) with Capcell Pak C18 column (250 mm×4.6 mm, Shiseido, Japan) was used for quantitative analysis. Elution was performed by gradient using a mixture of water and acetonitrile from 65 : 35 to 35 : 65 within 30 min (flow rate=1.0 mL/min). Effluent was monitored at 227 nm (paclitaxel) or 255 nm (internal standard) with the photo diode-array detector. Purity deter-

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minations of paclitaxel were made by using an internal standard assay to compare the paclitaxel content of the test material to the paclitaxel content of the reference paclitaxel. Purity values were calculated by comparing the response ratio determined for the test sample to that obtained for the reference paclitaxel, and concentrations for paclitaxel were estimated by reference to the paclitaxel peak area. Dried residue was redissolved in methanol and used for the quantitative analysis of paclitaxel. Authentic paclitaxel and internal standard, which is n-propyl paraben, was purchased from Sigma-Aldrich and used for the standard [13].

3. Preparation of Crude Extract for Fractional Precipitation

A crude extract for fractional precipitation was prepared from the biomass of *Taxus chinensis* cultures by the following steps: (i) organic solvent extraction of the biomass to obtain a crude extract, (ii) synthetic adsorbent treatment of the crude extract followed by filtration to remove adsorbent, and (iii) addition of hexane to the filtrate to obtain a crude precipitate [10]. The crude paclitaxel precipitate was filtered and dried for the fractional precipitation.

4. Fractional Precipitation

From the HPLC analysis, paclitaxel is the major taxoid component of the mixture and constitutes about 27.0% of the total sample on weight basis in crude extract from the hexane precipitation. The precipitate from the hexane precipitation was dissolved in methanol, dropped into distilled water with various pHs and left to stand at 4 ºC for 14 h to obtain paclitaxel precipitate. This is a fractional precipitation of paclitaxel by differences of solubility in methanol solution. After precipitation, the paclitaxel precipitate was filtered and dried at 40 ºC under reduced pressure.

5. Analysis of SEM (Scanning Electron Microscopy) and XRD (X-ray Diffractometer)

The morphology of paclitaxel from fractional precipitation was analyzed by SEM (scanning electron microscopy, JSM-6335F, Jeol, Japan) and X-ray diffractometer (D/WAX-3B, Rigaku, Japan). The XRD measurements were performed in the 5 to 40º 2θ range at a rate of $2^{\circ}2\theta$/min using CuKα radiation (45 kV, 40 mA) as X-ray source. The amount of each sample was about 50 mg.

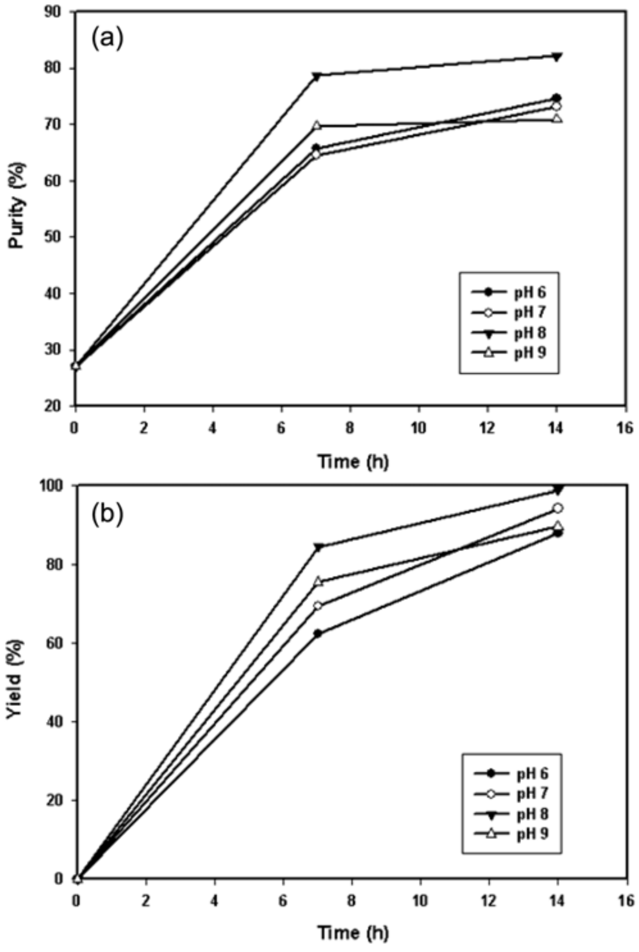

Fig. 1. Effect of pH on the purity (a) and yield (b) of paclitaxel in fractional precipitation. The methanol concentration in water, content in the crude extract, storage temperature, and storage time were 61.5% (v/v), 0.5% (w/v), 4 ºC, and 14 h, respectively.

RESULTS AND DISCUSSION

1. Effect of pH on Fractional Precipitation

Previously, we optimized most of the process variables in fractional precipitation, except distilled water pH [10,14]. The optimal methanol concentration in water, paclitaxel content in the crude extract, and storage temperature were 61.5% (v/v), 0.5% (pure paclitaxel basis, w/v), and 4 ºC, respectively. In this study, the distilled water pH, an important process variable in fractional precipitation, was optimized in terms of the yield and purity of paclitaxel for a fixed methanol concentration in water (61.5%, v/v), paclitaxel content in the crude extract (0.5%, w/v), and storage temperature (4 ºC). The crude extract was dissolved in methanol and dropped into distilled water to give 61.5% (v/v) methanol and left to stand at 4 ºC for 14 h to obtain a paclitaxel precipitate. This is a fractional precipitation of paclitaxel by differences of solubility in methanol solution. The greatest purity (~84.4%) and yield (~99.0%) were obtained at pH 8.0 with potassium phosphate buffer (Fig. 1). Several previous papers [5-9] deal with the isolation of paclitaxel from biomass as part of investigations into the identity of paclitaxel constituents. The resulting crude paclitaxel products typically contain between 1 and 4% paclitaxel by weight, and the final separation of paclitaxel is accomplished by using open column chromatography, HPLC, or a combination of the two methods. The low-resolution chromatography steps require such large amounts of solvent and chromatographic media that the process is impractical for scale-up to commercial operations [15-17].

The schematic diagram of the fractional precipitation and the results obtained for this pre-purification of paclitaxel from plant cell cultures is summarized in Fig. 2. The optimal pH was 8.0 at fixed methanol concentration in water, paclitaxel content in the crude extract, and storage temperature were 61.5% (v/v), 0.5% (pure paclitaxel basis, w/v), and 4 ºC, respectively. The crude paclitaxel was efficiently pre-purified, increasing in purity from 27.0% to over 84.4% with a yield 99.0% by fractional precipitation. Fractional precipitation was performed to obtain paclitaxel with a high purity, which is adjustable to apply to the column employed in a subsequent HPLC purification step. The purity level (~84.4%) in crude paclitaxel after fractional precipitation ensures that a minimum of material enters the HPLC purification process, thereby minimizing costs. This method

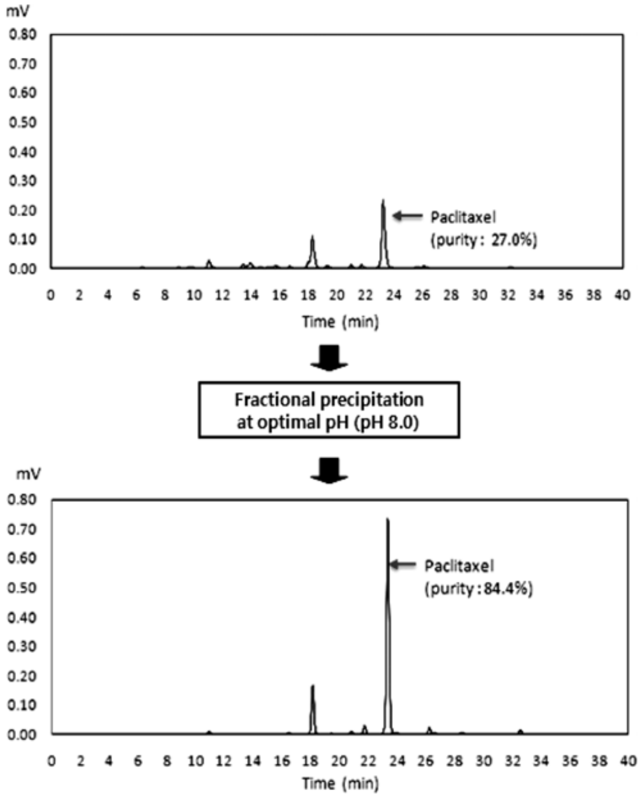

Fig. 2. Schematic diagram of the fractional precipitation and the results obtained for the pre-purification of paclitaxel from biomass. The methanol concentration in water, paclitaxel content in the crude extract, storage temperature, and storage time were 61.5% (v/v), 0.5% (w/v), 4 °C, and 14 h, respectively.

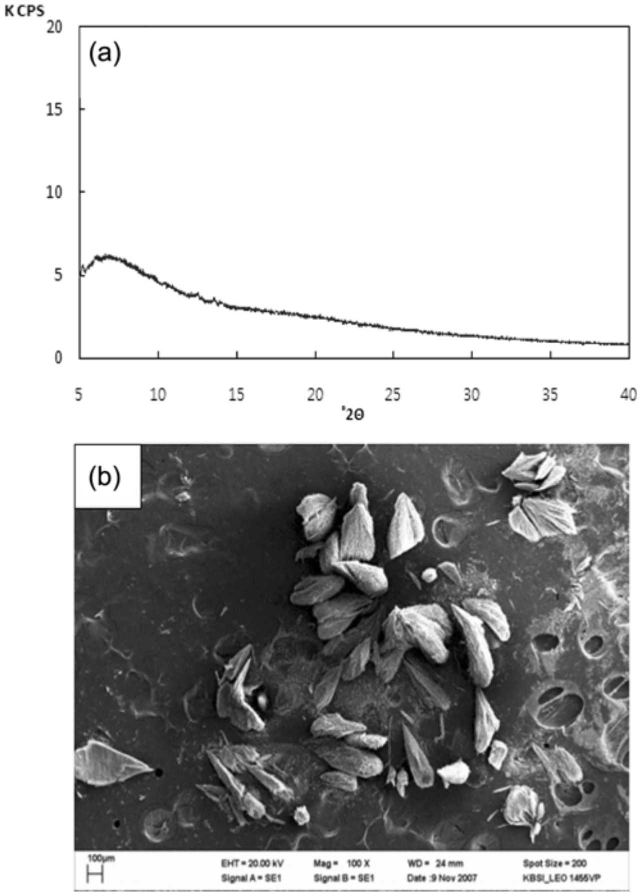

Fig. 3. XRD (a) and SEM (b) pictures of precipitate from fractional precipitation at pH 8.0.

is a simple and efficient procedure for the pre-purification of paclitaxel from plant cell cultures. Especially, it is very efficient for removing the polar impurities in the crude paclitaxel. This process allows for rapid separation of paclitaxel from interfering compounds and dramatically reduces solvent usage compared to alternative processes. This pre-purification process serves to minimize the size and complexity of the HPLC operations for paclitaxel purification.

2. Morphology Analysis of Precipitate

To identify the morphology of the paclitaxel from fractional precipitation at pH 8.0, XRD and SEM data of the samples were compared with those of the reference [18,19]. Fig. 3(a) shows the XRD pattern of the precipitate. In comparison with the previous study, it can be easily noticed that the XRD pattern of precipitate was identical to that of amorphous paclitaxel. For this precipitate, we could see no meaningful peaks in the XRD pattern. Therefore, this precipitate is amorphous state paclitaxel. The SEM analysis of the above sample also gave us the same result (Fig. 3(b)). Also, the particle size distribution of dried precipitate was a range of approximately 119-411 µm. The amorphous form of pharmacologically active materials has received considerable attention because, in theory, this form represents the most energetic solid state of a material, and thus it should provide the biggest advantage in terms of solubility and bioavailability [20,21]. Additionally, it may provide significant changes from the usual crystalline form in terms of its mechanical properties, such as elastic modulus.

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