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Improved fractional precipitation method for purification of paclitaxel

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ABSTRACT

This study investigated changing the methanol/water ratio during fractional precipitation of paclitaxel, and adding all the distilled water at room temperature, followed mixing for an additional 10 min. When the methanol/water ratio was 50:50, 40:60, and 30:70 (v/v), the paclitaxel yield was 42.0%, 84.3%, and 92.0%, respectively. When using a methanol/water ratio of 50:50 (v/v), a similar high purity and yield of paclitaxel to the case of storing at a low temperature was achieved when adding all the distilled water at room temperature, followed by additional mixing for 10 min and further mixing at room temperature during fractional precipitation. Thus, additional mixing after adding all the distilled water is confirmed as important during fractional precipitation. Furthermore, the present results show that a high yield of high-purity paclitaxel is possible with additional mixing at room temperature after adding all the distilled water, which is significantly more economical than the existing method of storing at a low temperature for a long time after adding all the distilled water during fractional precipitation.

1. Introduction

Paclitaxel is a diterpenoid anticancer agent found in the bark of the yew tree. Its chemical structure was established by Wani et al. [1] in 1971 and its anticancer mechanism revealed by Schiff et al. [2] in 1979. It has been approved by the US Food and Drug Administration as a treatment for ovarian cancer, breast cancer, Kaposi's sarcoma, and non-small cell lung cancer (NSCLC). Paclitaxel is currently the most widely used anticancer drug. The demand for this drug is expected to increase steadily; its indications, which include acute rheumatoid arthritis and Alzheimer's disease, are expanding continuously, and clinical tests for combined prescription with other therapies are being conducted [3]. The major production methods of paclitaxel include direct extraction from yew trees [4], semi-synthesis by chemically binding a side chain based on obtaining a precursor (baccatin III, 10-deacetylbaccatin III, 10-deacetylpaclitaxel, etc.) from the leaves of yew trees [5], and a plant cell culture in a bioreactor following a seed culture from a callus induced from a yew tree [6,7]. Among these methods, plant cell culture has the merit of mass-producing a certain quality of paclitaxel because stable production is possible within the bioreactor without being affected by external factors such as climate and environment [3].

To obtain a high purity (>98%) of paclitaxel from a plant cell culture, several separation and purification steps are required. The paclitaxel is normally initially extracted from the biomass (plant cells containing paclitaxel) as the raw material using an organic solvent, which is then followed by a pre-purification process and final purification process. In particular, the pre-purification process has a significant impact on the cost of final purification [8–10]. Highly expensive chromatography methods can be used for pre-purification or the crude extract can be directly processed using high performance liquid chromatography (HPLC) without pre-purification; the latter approach entails serious problems with cost as well as with scaling-up and industrial mass production. Usually, the purity of paclitaxel extracted from biomass using an organic solvent is around 0.5%, and it is still very low, under 10%, even after a simple pre-purification process. If the sample then proceeds directly to final purification by HPLC, a large amount of the organic solvent is consumed, the lifetime of the column packing material (resin) is reduced, and the throughput is decreased. Therefore, the cost of final purification, especially when HPLC is used, can be reduced if the purity of the pre-purified sample is increased as much as possible [11].

Among the various pre-purification methods, fractional precipitation can provide a high yield of high-purity paclitaxel easily and simply using the difference in the solubility of the anticancer agent paclitaxel. The first study on high-purity (>50%) paclitaxel using fractional precipitation was reported in 2000 [12], a study on the optimal methanol content for fractional precipitation was conducted in 2002 [13], and a study on the storing temperature

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after fractional precipitation was performed in 2006 [14], all of which facilitate high-purity and high-yield paclitaxel. However, the main drawback of fractional precipitation is the long time (~3 days) and low temperature (0–4 °C) required for precipitation, thereby limiting the mass production of paclitaxel from an economical aspect. Therefore, further studies have been conducted in 2009–2013 to improve the efficiency of fractional precipitation by increasing the surface area per working volume (S/V) using surface area-increasing materials, such as glass beads or ion exchange resin [10,11,15,16], along with a study to enhance the efficiency of precipitation by investigating the effect of the reactor type, crude extract purity and pure paclitaxel content [17,18]. As a result, the long time required for fractional precipitation has been shortened, yet this is still not enough for the mass production of paclitaxel. Accordingly, this study presents a method for dramatically improving the precipitation time and storing temperature required for fractional precipitation. As such, the purity and yield of fractional precipitation are investigated when changing the methanol/water ratio in the fractional precipitation solution, varying the crude extract purity, and adjusting the mixing and storing temperature during fractional precipitation.

2. Materials and methods

2.1. Plant materials and culture conditions

A suspension of cells originating from *Taxus chinensis* was maintained in darkness at 24 °C with shaking at 150 rpm. The cells were cultured in a modified Gamborg's B5 medium [19] supplemented with 30 g/L sucrose, 10 mM naphthalene acetic acid, 0.2 mM 6-benzylaminopurine, 1 g/L casein hydrolysate, and 1 g/L 2-(N-morpholino)ethanesulfonic acid. The cell cultures were transferred to a fresh medium every 2 weeks. During prolonged culture for production purposes, 4 mM $AgNO_3$ was added at the initiation of the culture as an elicitor, and maltose (1 and 2%, w/v) added to the medium on day 7 and 21, respectively [7]. Following cultivation, the biomass was recovered using a decanter (CA150 Clarifying Decanter; Westfalia) and high-speed centrifuge (BTPX 205GD-35CDEFP; Alfa-Laval). The biomass was provided by the Samyang Genex Company, South Korea.

2.2. Sample preparation for fractional precipitation

Biomass was extracted with methanol in a ratio of 1:1 (w/v) at room temperature for 30 min. The mixture was filtered under vacuum in a Buchner funnel through filter paper. Extraction was repeated at least four times. Each methanol extract was collected, pooled and concentrated under vacuum at 635 mm Hg and 40 °C to reduce the volume to 30% of the original. Methylene chloride (25% of concentrated methanol extract) was added and liquid–liquid extraction was performed three times for 30 min. Paclitaxel was collected into the lower methylene chloride layer for concentration, followed by vacuum filtration with filter paper (150 mm; Whatman) and drying. The dried crude extract obtained by liquid–liquid extraction was dissolved in methylene chloride at a ratio of 20% (v/w), and commercial adsorbent sylopute (Fuji Silysia Chemical Ltd., Japan) added at a ratio of 100% (w/w) with the dried crude extract. The mixture was agitated for 30 min at 40 °C, then filtered. The filtrate was dried at 30 °C under vacuum and subjected to a hexane precipitation process. Dried extract was dissolved in methylene chloride and dropped in hexane (methylene chloride/hexane = 1/10, v/v) to induce precipitation to remove the non-polar impurities. After hexane precipitation, the paclitaxel precipitate was obtained by filtration and it was dried in a vacuum oven (UP-2000; EYELA) at 35 °C for 24 h. Various types of crude extract (purity: 17.6%, 21.3%, 27.6%, 32.6%, 42.0%) obtained by hexane precipitation were used as samples for the fractional precipitation.

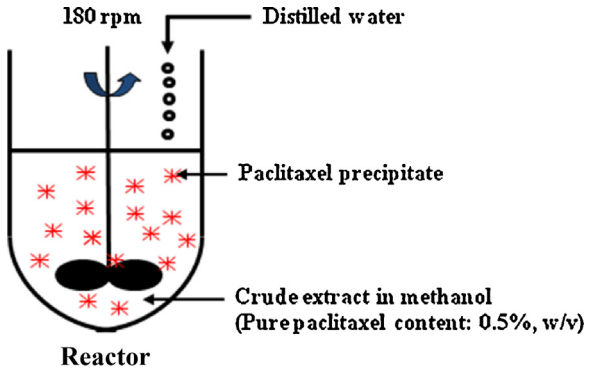

Fig. 1. Schematic diagram of fractional precipitation for pre-purification of paclitaxel.

2.3. Fractional precipitation

Fig. 1 shows a schematic diagram of the fractional precipitation process using the difference in solubility of paclitaxel in methanol solution. For the fractional precipitation, the samples (purity: 17.6%, 21.3%, 27.6%, 32.6%, 42.0%) were dissolved in methanol (pure paclitaxel basis: 0.5%, w/v), while distilled water was added under agitation (180 rpm) until the methanol/water ratio became 50:50, 45:55, 40:60, 35:65, 30:70, and 20:80 (v/v) at room temperature. After adding the distilled water, the solution was mixed for 10 min. To investigate the effect of the storing temperature after mixing (10 min), the storing temperature was changed (4 °C, room temperature) to determine the purity and yield of paclitaxel depending on the precipitation time (0, 0.5, 1, 2 h). In addition, to confirm the effect of additional mixing after the initial mixing (10 min), the fractional precipitation was conducted with different additional mixing periods (0, 0.5, 1, 2 h) at room temperature. After the precipitation, the precipitate was obtained through filtration with filter paper (Whatman Grade 4, 20–25 μm particle retention, 150 mm diameter) and was vacuum dried for 24 h at 35 °C. The purity and yield of the dried precipitate were analyzed using HPLC.

2.4. Paclitaxel analysis

The dried residue was redissolved in methanol for a quantitative analysis using an HPLC system (Waters, USA) with a Capcell Pak C18 column (250 mm × 4.6 mm; Shiseido). The elution was performed based on a gradient using a distilled water–acetonitrile mixture varying from 65:35 to 35:65 within 40 min (flow rate = 1.0 mL/min). The injection volume was 20 μL, and the effluent was monitored at 227 nm using a UV detector. Authentic paclitaxel (purity: 97%) was purchased from Sigma–Aldrich and used as the standard [11].

3. Results and discussion

3.1. Effect of methanol/water ratio

Fractional precipitation is a very simple method for the purification of paclitaxel in high yield that relies on solubility differences. However, the existing fractional precipitation takes a long time (~3 days) and is conducted at a low temperature (0–4 °C), thereby limiting its application for the economical mass-production of paclitaxel [12–14]. To improve these problems, the fractional precipitation was performed using different methanol/water ratios at 50:50, 40:60, and 30:70 (v/v) with the crude extract (purity: 21.3%). The effect on the fractional precipitation (purity, yield,

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