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Precipitation characteristics of paclitaxel in solvent systems with different ion exchange resins

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Abstract−We systematically examined the effect of the solvent system (methanol/water or acetone/pentane) on precipitation characteristics and mechanisms in the increased surface area precipitation (ISAP) with different ion exchange resins for the purification of paclitaxel. When Amberlite IRA 400Cl was added to increase the surface area, the acetone/pentane system was found to be more effective than the methanol/water system in terms of paclitaxel purity. The addition of surface area-increasing materials increased the yield in the methanol/water system, whereas it decreased the yield in the acetone/pentane system. Precipitates in the methanol/water system were needle-shaped or star-shaped, spreading from the central nucleus along the growing branches, while precipitates in the acetone/pentane system were disk shaped, branching out from around the nucleus. When Amberlite IRA 400Cl was added, it was possible to obtain smaller paclitaxel precipitates in the acetone/pentane system than in the methanol/water system.

Key words: Paclitaxel, Precipitation, Characteristics, Solvent System, Ion Exchange Resin

INTRODUCTION

Paclitaxel is a diterpenoid anticancer agent that was discovered in the bark of the yew tree. It is currently the most important FDA-approved anticancer drug used for the treatment of ovarian cancer, breast cancer, Kaposi's sarcoma and non-small cell lung cancer [1, 2]. Its application to the treatment of diseases including rheumatoid arthritis and Alzheimer's continues to be expanded, and clinical tests of its combined prescription with many other treatment methods are underway; thus, the demand for paclitaxel is expected to increase continuously in the future [3].

There are several methods of paclitaxel production. The first is direct extraction from the yew tree [4], where both acquiring a continuous supply of raw materials and extraction/purification are difficult. Furthermore, this approach requires the harvest of yew trees, which are a protected species in some areas. In the second method, precursors (baccatin III, 10-deacetylbaccatin III, 10-deacetylpaclitaxel, etc.) can be obtained from yew leaves and their side chains chemically bound via the semi-synthesis method [5]. Because this method likewise requires obtaining precursors from yew trees, it has problems identical to those of direct extraction. In a third method, calluses are induced from yew trees, and plant cells are cultured in a bioreactor through seed culture [6]. Among these methods, plant cell culture enables the stable mass production of paclitaxel of consistent quality in a bioreactor without the influence of external factors such as climate and environment. To obtain high purity paclitaxel from plant cell cultures, several separation and purification steps are required. Typically, after biomass (i.e., plant cells containing paclitaxel) is recovered from the cell culture broth and is extracted with an organic solvent, pre-purification and final purification processes follow. Among these processes, pre-purification in particular has a significant impact on the cost of final purification [7-9]. Processes reported in the literature [10-12] either use highly expensive chromatographic methods for pre-purification or directly process the crude extract using high performance liquid chromatography (HPLC) without pre-purification. If these methods are introduced, a large amount of organic solvent is consumed, the lifetime of the column packing material (resin) is reduced, and 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 [13].

In 2000 and 2005, an efficient pre-purification method was developed that enabled a high purity (>50%) of paclitaxel to be obtained by methanol/water fractional precipitation [13] and acetone/pentane precipitation [14], respectively. Recently, a method was also reported that could decrease the time required for precipitation by increasing the surface area per working volume (S/V) using a surface area-increasing material [15-18]. However, the examination of precipitation characteristics and mechanisms in different solvent systems with surface area-increasing materials is considerably inadequate. Therefore, we systematically examined the effect of the solvent system (methanol/water or acetone/pentane) on precipitation characteristics and mechanisms in the increased surface area precipitation (ISAP) with different ion exchange resins for the purification of paclitaxel. The results are expected to contribute considerably to the improvement of precipitation efficiency in the purification process for the anticancer agent paclitaxel.

MATERIALS AND METHODS

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 modified Gamborg's B5 medium [19] supplemented with 30 g/L sucrose, 10 mM naphthalene acetic acid, 0.2 mM 6-

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benzylaminopurine, 1 g/L casein hydrolysate, and 1 g/L 2-(N-morpholino) ethanesulfonic acid. Cell cultures were transferred to fresh medium every two weeks. During prolonged culture for production purposes, 4 mM $AgNO_3$ was added at the initiation of the culture as an elicitor, and 1% and 2% (w/v) maltose were added to the medium on days 7 and 21, respectively [14]. Following cultivation, biomass was recovered with a decanter (CA150 Clarifying Decanter; Westphalia, Germany) and a high-speed centrifuge (BTPX 205GD-35CDEFP; Alfa Laval, Sweden). The biomass was provided by Samyang Genex Company, South Korea.

2. Paclitaxel Analysis

Dried residue was redissolved in methanol for quantitative analysis by using an HPLC system (Waters, USA) with a Capcell Pak C_{18} column (250 mm×4.6 mm; Shiseido, Japan). Elution was performed in 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 with a UV detector. Authentic paclitaxel (purity: 97%) was purchased from Sigma-Aldrich and used as a standard [20]. Each sample was analyzed in triplicate.

3. Sample Preparation for Precipitation

Biomass was mixed with methanol and stirred at room temperature for 30 min. The mixture was filtered under vacuum in a Buchner funnel through filter paper, where the biomass was preferably added to methanol at a ratio of 100%. 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. During the extraction, polar impurities were dissolved in the methanol layer (top phase). After this layer was removed, the methylene chloride layer (bottom phase) containing paclitaxel was collected and concentrated/dried under reduced pressure. Dried crude extract was dissolved in methylene chloride at a ratio of 20% (v/w) and then sylopute (Fuji Silysia Chemical Ltd., Japan), an adsorbent, was added at a ratio of 50% (w/w) for removal of biomass-derived tar and waxy compounds. The mixture was agitated for 30 min at 40 °C, and then filtered. The filtrate was dried at 30 °C under reduced pressure and then subjected to hexane precipitation. Dried crude extract was dissolved in methylene chloride, which was dropped into hexane to remove non-polar impurities with induction of precipitation (methylene chloride/hexane =1 : 10, v/v). After hexane precipitation, the paclitaxel precipitate was obtained through filtration and then dried in a 35 °C vacuum oven (UP-2000; EYELA) for 24 hr [7,15]. The dried material (purity: 41.8%) obtained from the hexane precipitation was subjected to ISAP in methanol/water and acetone/pentane.

4. ISAP in Methanol/Water and Acetone/Pentane

For methanol/water fractional precipitation, the crude extract (purity of paclitaxel: 41.8%) obtained from hexane precipitation was dissolved in methanol (pure paclitaxel basis: 0.5%, w/v), and distilled water was added dropwise with stirring (180 rpm) until the methanol concentration reached 61.5% [13,15,16]. The reactor size and working volume were 10 and 2.72 mL, respectively. For acetone/pentane precipitation, the same sample (crude extract) used for methanol/water fractional precipitation was dissolved in acetone (pure paclitaxel basis: 0.5%, w/v) and n-pentane was added dropwise at a ratio of 1 : 7 (acetone/pentane, v/v) under agitation (335 rpm) [14]. The reactor size and working volume were 20 and 9.57 mL, respectively. The cation exchange resins Amberlite IR 120Plus and IRC 76 and the anion exchange resins Amberlite IRA 400Cl and IRA 67 (Sigma-Aldrich), which were the typical resins used to increase S/V in previous studies [16-18], were added separately without agitation to increase the S/V of the reaction solution. Ion exchange resin was used in the experiment after drying for 1 day at 35 °C. The experiment was conducted with the S/V of the reacting solution fixed at the optimal level of 0.428 mm^{-1}, as suggested in a previous study [15]. The S/V was calculated for each ion exchange resin as follows:

$$S/V[mm^{-1}] = [\text{total surface area of resin } (mm^2)/\text{working volume } (mm^3)] \quad (1)$$

To obtain paclitaxel precipitate after the addition of the surface area-increasing material, the preparation was stored for 24 hr at a low temperature (4-7 °C). After precipitation, the paclitaxel precipitate was filtered (Whatman Grade 4, 150 mm diameter) and vacuum dried (635 mm Hg, 35 °C) for 24 hr.

5. Analysis of Paclitaxel Precipitate

The paclitaxel precipitate was visualized during the precipitation process with an SV-35 Video Microscope System (Some Tech, Korea) at high magnification (100×) [15]. The size and shape of paclitaxel particles in dynamic images were verified with IT-Plus software (Some Tech, Korea).

RESULTS AND DISCUSSION

1. Effect of Solvent System on ISAP Efficiency

Precipitation is a very simple method of purifying the anticancer agent paclitaxel efficiently by utilizing the difference in solubility of paclitaxel in solvents. It is typically applied to the production of high purity paclitaxel with high yield in the purification step. Two kinds of solvent systems (methanol/water or acetone/pentane) for precipitation have been reported in the literature [13,14]. Herein, the precipitation characteristics (purity, yield, precipitation time, and shape and size of precipitate) and mechanisms of the two solvent systems in the ISAP process were systematically examined. The precipitation was performed using various types of ion exchange resins (Amberlite IR 120plus, Amberlite IRC 76, Amberlite IRA 400Cl, Amberlite IRA 67) to increase the surface area; paclitaxel precipitate formed using each of the resins in the methanol/water system while precipitate formed only with Amberlite IRA 400Cl in the acetone/pentane system. As the precipitation time increased, the purity of paclitaxel increased in both solvent systems, and when S/V was increased using ion exchange resins, the purity of paclitaxel was higher than that of the control without the addition of surface area-increasing materials (Fig. 1). Little effect of the solvent system on paclitaxel purity was exhibited in the control. When Amberlite IRA 400Cl was added to the two solvent systems, the acetone/pentane system was determined to be more efficient since it enabled paclitaxel of 75.4% purity to be obtained, whereas a purity of 66.7% was achieved in methanol/water for the given precipitation time (18 hr).

For the methanol/water system, precipitation occurred successfully with every ion exchange resin used in the experiment. In particular, when S/V was increased using Amberlite IRA 400Cl, the highest yield (>75%) of paclitaxel was obtained (Fig. 2(a)). On the other

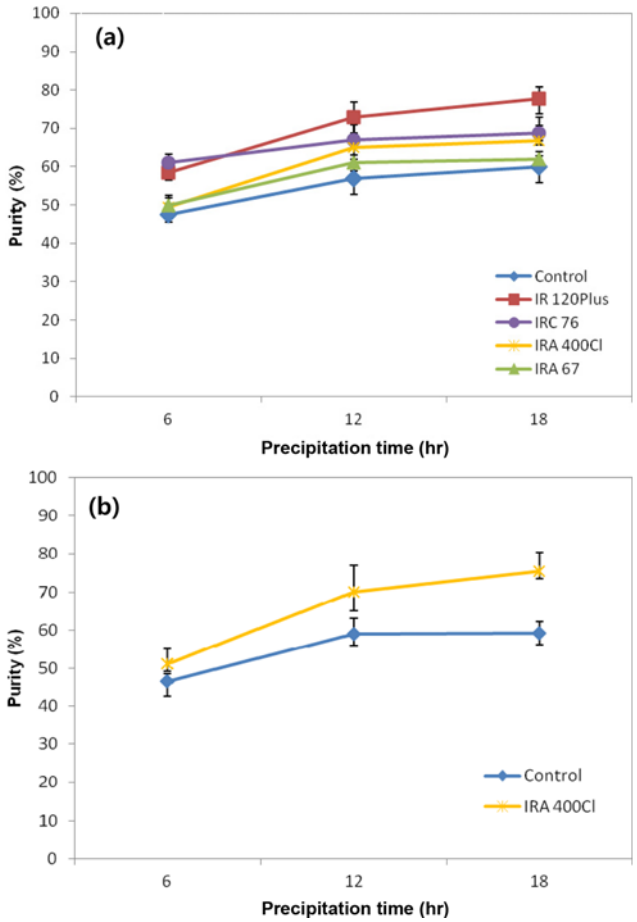

Fig. 1. Effect of increased surface area per working volume (S/V: 0.428 mm^{-1}) on the purity of paclitaxel from methanol/water (a) and acetone/pentane (b) precipitation.

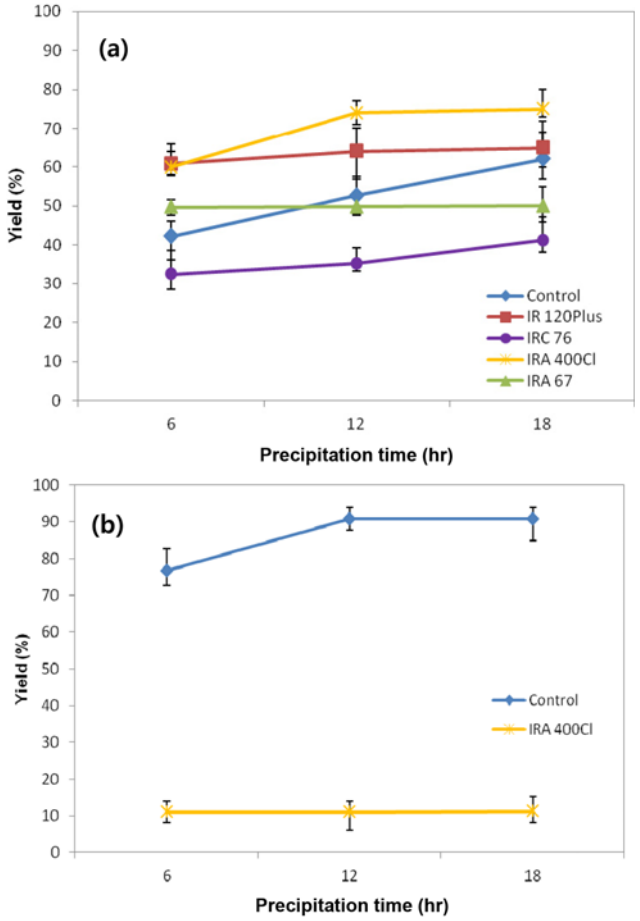

Fig. 2. Effect of increased surface area per working volume (S/V: 0.428 mm^{-1}) on the yield of paclitaxel from methanol/water (a) and acetone/pentane (b) precipitation.

hand, only the addition of Amberlite IRA 400Cl resulted in the formation of paclitaxel precipitate (paclitaxel yield: 11.2%) in the acetone/pentane system (Fig. 2(b)). When S/V was increased using Amberlite IRA 400Cl, the yield of paclitaxel was dramatically decreased in comparison with the control (yield >90%). When surface area-increasing materials were added, the yield of paclitaxel was higher in the methanol/water system than in the acetone/pentane system, whereas, when surface area-increasing materials were not added, the acetone/pentane system was more effective.

For a given precipitation time, a higher yield was obtained in the methanol/water system when the S/V of the reacting solution was increased using Amberlite IRA 400Cl compared to the control. However, the yield was decreased significantly in the acetone/pentane system when the S/V of the reacting solution was increased using Amberlite IRA 400Cl compared to the control. Given this, the experimental results for the solvent system effect on the yield of paclitaxel can be hypothetically explained as shown in Fig. 3. This result suggests that in the different solvents, differences in the value of the proton-acceptor solubility parameter affected the yield of paclitaxel. When its value is higher, the affinity of paclitaxel for the solvent is high, and the value is higher in methanol/water than in acetone/pentane [21]. Therefore, in the methanol/water system, with its higher value for the proton-acceptor solubility parameter, paclitaxel has a

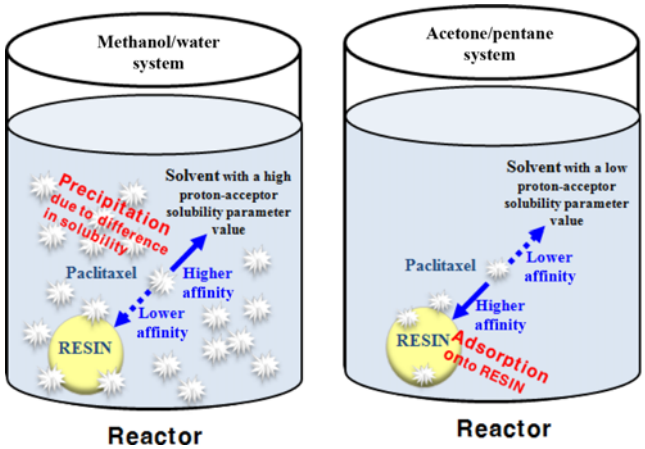

Fig. 3. Hypothetical mechanisms proposed to explain the precipitation characteristics of paclitaxel in different solvent systems.

higher affinity for the solution than for Amberlite IRA 400Cl. At the end of the experiment, it seems that paclitaxel within the solution formed more precipitates according to differences in solubility. On the other hand, in the acetone/pentane system, with its relatively lower value for the proton-acceptor solubility parameter, paclitaxel

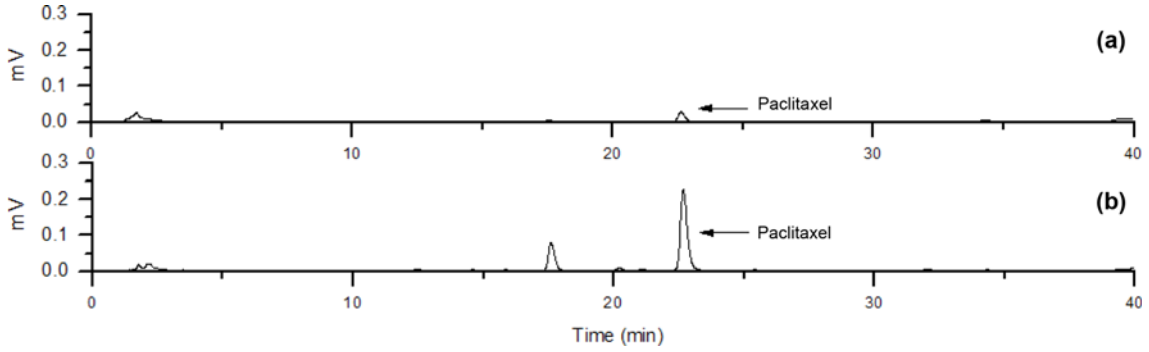

Fig. 4. HPLC chromatograms of Amberlite IRA 400Cl washing solution after precipitation. (a): methanol/water system; (b): acetone/pentane system.

has a lower affinity for the solution than for Amberlite IRA 400Cl. In turn, paclitaxel was adsorbed onto the surface area-increasing material. Therefore, the yield of paclitaxel precipitate seems to decrease. To investigate the degree of paclitaxel adsorption on Amberlite IRA 400Cl, the resin was collected after precipitation and washed with methanol for HPLC analysis. As shown in Fig. 4, larger peaks for impurities including paclitaxel were observed in the acetone/pentane system than in the methanol/water system; in particular, the evidence suggests that paclitaxel was more adsorbed onto Amberlite IRA 400Cl. As a result, when increasing S/V with the addition of Amberlite IRA 400Cl, the acetone/pentane system was more effective than the methanol/water system in terms of the purity of paclitaxel. Meanwhile, the results suggest that the methanol/water system was more effective than the acetone/pentane system in terms of the yield of paclitaxel. Thus, the different types of solvent used for the precipitation process affect the affinity of paclitaxel for the solvent differently. As a result, differences in the solvent system used affect the purity and yield of paclitaxel.

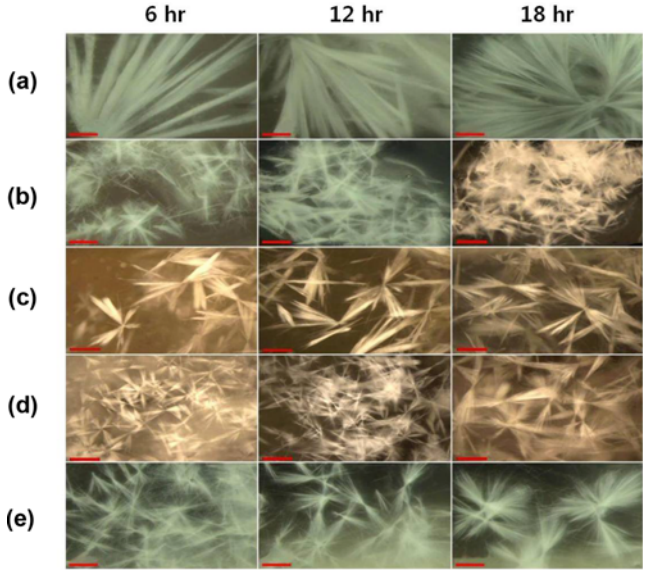

Fig. 5. Electron micrograph of paclitaxel precipitate from methanol/water fractional precipitation at 18 hr. Control (a); cation exchange resins Amberlite IR 120Plus (b), and Amberlite IRC 76 (c); and anion exchange resins Amberlite IRA 400Cl (d) and Amberlite IRA 67 (e). Scale bar indicates 10 μm.

2. Change in Shape and Size of Paclitaxel Precipitate

Differences in the shape and size of the paclitaxel precipitate in the two different solvent systems in the ISAP process were examined with an electron microscope. For the methanol/water system, precipitates were needle-shaped or star-shaped, spreading from the central nucleus along the growing branches (Fig. 5). Meanwhile, the precipitate in the acetone/pentane system was identified as disk shaped, branching out from around the nucleus (Fig. 6). Depending on the solvent system, the surface energy of crystallization differed and the crystallized shape changed [22]. That is, with high surface energy, the crystal face grew quickly but unstably, while the crystal face with low surface energy grew slowly and stably. The results show that in the methanol/water and acetone/pentane systems, the size of paclitaxel precipitates decreased significantly in the presence of surface area-increasing materials (ion exchange resins) compared to the control for a given precipitation time (Fig. 7). The surface area-increasing material thus served as an effective steric barrier hindering the growth of paclitaxel particles. In the case of ISAP with the methanol/water system, the size of paclitaxel particle decreased with increasing precipitation time, which could be attributed to frequent collisions due to the growth of particles within a limited volume in the reactor. When Amberlite IRA 400Cl was added, smaller particles of paclitaxel were obtained more effectively in the acetone/pentane system than in the methanol/water system. Likewise, the size differences of paclitaxel precipitate in the two solvent systems seemed to be attributed to the different values of the proton-acceptor solubility parameter of the solvent [21]. In the control without

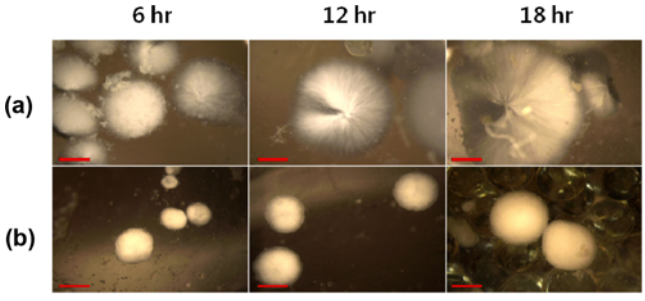

Fig. 6. Electron micrograph of paclitaxel precipitate from acetone/pentane precipitation at 18 hr. Control (a) and anion exchange resin Amberlite IRA 400Cl (b). Scale bar indicates 10 μm.

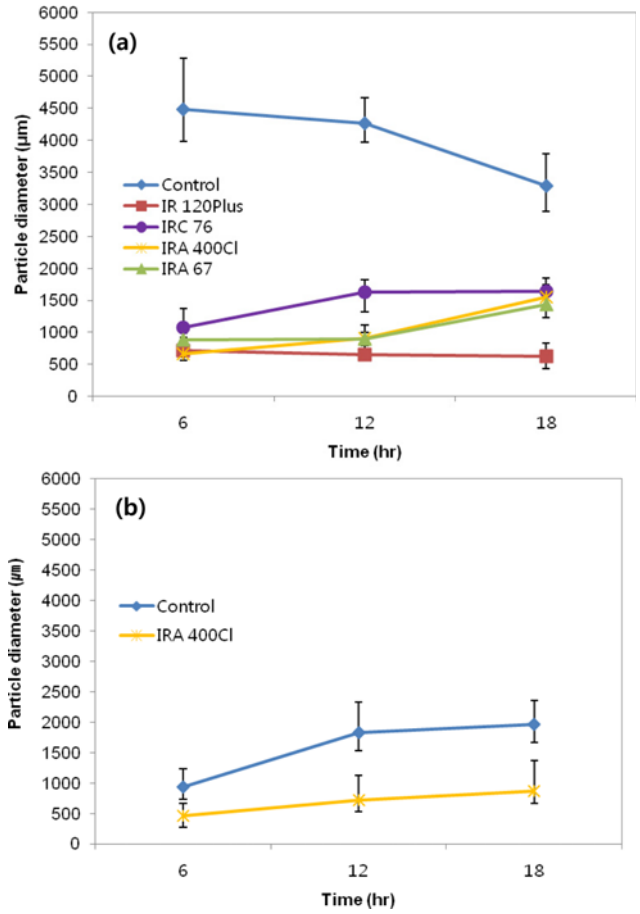

Fig. 7. Effect of increased surface area per working volume (S/V: 0.428 mm^{-1}) on the size of paclitaxel precipitates over time in methanol/water (a) and acetone/pentane (b) precipitation.

the addition of a surface area-increasing material, smaller particles of paclitaxel were collected from the acetone/pentane system with its low value for the proton-acceptor solubility parameter, and relatively large sized paclitaxel particles were obtained from the methanol/water system with its high value for the proton-acceptor solubility parameter. This result is due to the fact that as the affinity between the solvent and paclitaxel particles increased, regional supersaturation spots were generated to form a larger particle paclitaxel [21]. This phenomenon has been confirmed in the production process for the drug carrier poly (D,L-lactide-*co*-glycolide), in which changes in solvent were able to control the polymeric nanoparticle size efficiently [23]. As a result, it has been determined that the size of precipitate is highly affected by the types of solvent used in the precipitation process. If the particle size is smaller, it is easy to remove residual water and solvent during the drying process after purification. From this point of view, paclitaxel with reduced particle size due to the addition of surface area-increasing materials during the precipitation process is believed to be useful in respect to the usability of the drug [14].

CONCLUSIONS

We examined the precipitation characteristics and mechanisms in detail depending on the increase in S/V within a precipitator during methanol/water fractional precipitation and acetone/pentane precipitation processes to separate and purify the anticancer drug paclitaxel from plant cell cultures. Different types of ion exchange resins (Amberlite IR 120Plus, Amberlite IRC 76, Amberlite IRA 400Cl, Amberlite IRA 67) were used to increase the surface area in the precipitation process, and precipitates successfully formed in the presence of all of the ion exchange resins in the methanol/water system, whereas precipitates formed only with Amberlite IRA 400Cl in the acetone/pentane system. Regardless of the solvent system used, when ion exchange resins were added to increase the surface area, the purity of paclitaxel increased compared to the control in their absence. When Amberlite IRA 400Cl was added, the acetone/pentane system was more effective for obtaining purified paclitaxel than the methanol/water system. Adding surface area-increasing materials increased the yield of paclitaxel in the methanol/water system, but decreased the yield in the acetone/pentane system. Precipitates in the methanol/water system were needle-shaped or star-shaped, spreading from the central nucleus along the growing branches, whereas precipitates in the acetone/pentane system were disk shaped, branching out from around the nucleus. The evidence shows that when ion exchange resins were added to increase the surface area, the size of paclitaxel precipitates was considerably decreased compared to the control in the methanol/water and acetone/pentane systems for the given precipitation time. When Amberlite IRA 400Cl was added, smaller precipitate particles were more obtainable in the acetone/pentane system than the methanol/water system. Therefore, it is confirmed that the choice of solvent system affects the purity, yield, and shape and size of precipitates of paclitaxel in the precipitation process. These results are expected to contribute considerably to the improvement of precipitation efficiency in the purification process for the anticancer agent paclitaxel.

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