Korean J. Chem. Eng., 39(1), 58-62 (2022)
DOI: 10.1007/s11814-021-0959-1

pISSN: 0256-1115
eISSN: 1975-7220

RAPID COMMUNICATION

Negative pressure cavitation fractional precipitation for the purification of paclitaxel from *Taxus chinensis*

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(*Received 15 June 2021 • Revised 6 September 2021 • Accepted 14 September 2021*)

Abstract−The precipitation efficiency of paclitaxel from *Taxus chinensis* was remarkably improved through negative pressure cavitation fractional precipitation. When paclitaxel was precipitated under a negative pressure of −200 mmHg, almost all of the paclitaxel (>97%) could be recovered in a short operation time (1 min). The precipitation rate constant was calculated using the JMAK equation for kinetic analysis. The rate constant in the case of negative pressure (−50 to −200 mmHg) cavitation fractional precipitation increased by 2.147-6.046 times compared to fractional precipitation without negative pressure. The change of activation energy by the negative pressure (−50 to −200 mmHg) was also calculated using the Arrhenius equation. The activation energy changes were −1,767 to −4,161 J/mol, implying that the activation energy could be reduced by introducing negative pressure, resulting in an increased precipitation rate. In addition, the application of negative pressure reduced the size of the precipitate by 3.3 times and increased the diffusion coefficient of paclitaxel by 4.4 times.

Keywords: Paclitaxel, Fractional Precipitation, Negative Pressure, Cavitation, Kinetics

INTRODUCTION

Paclitaxel (molecular formula: $C_{47}H_{51}NO_{14}$) is a diterpenoid alkaloid found in the bark of yew trees. This molecule improves the stability of microtubules, thus inhibiting depolymerization to induce apoptosis of cancer cells. Therefore, the unique pharmacological effect of paclitaxel is widely used to treat ovarian cancer, breast cancer, head and neck cancer, Kaposi's sarcoma, and non-small cell lung cancer [1-3]. The demand for paclitaxel is expected to grow with the further expansion of its indications and the development of new treatment methods [4-6]. Paclitaxel is mainly produced through direct extraction, semi-synthesis using its precursors and plant cell culture from the callus derived from yew plants [7-9]. Among these production methods, plant cell culture has merit as it is less affected by the external factors, such as environment and climate, and has stable production with uniform quality in a bioreactor [9,10].

In the plant cell culture, paclitaxel is accumulated mainly in biomass and is produced through several steps of extraction, pretreatment, and final purification. In particular, pretreatment greatly affects the final purification cost [11,12]. Therefore, the purification cost, specifically through HPLC, can only be reduced when the sample purity is increased through pretreatment.

Fractional precipitation is a simple and efficient pretreatment method based on the solubility difference of paclitaxel [9]. The fractional precipitation of plant-derived paclitaxel was reported for the first time in 2000 [13] and high purity paclitaxel could be obtained with a high yield by optimizing the fractional precipitation conditions (solvents, temperature, and properties of sample) [14,15]. However, fractional precipitation operates at low temperatures over a long time so it has limited applications in the mass production of paclitaxel from an economic point of view. Numerous studies have attempted to improve the precipitation efficiency by increasing the surface area per working volume (S/V) through the introduction of glass beads or ion exchange resins [16,17]. Research was also conducted to improve the precipitation efficiency via adjustment and control of the impurity levels of the sample [17]. Although the long precipitation time has been shortened to some extent through these studies, it is still insufficient for the mass production of paclitaxel. In 2014, the precipitation time was shortened by controlling the polarity of the precipitation solution (methanol-distilled water mixture), but the purity of paclitaxel was impaired by adding excessive amounts of distilled water [18]. Recently, fractional precipitation using ultrasonic waves contributed to remarkable improvement in the long precipitation time [10]. By introducing ultrasonic waves during precipitation, the nucleating rate was increased, resulting in a decrease in precipitation time. Such a result was attributed to ultrasonic cavitation, wherein cavitation bubbles become unstable from compression and expansion and finally collapse to create microjets, intense localized heating, and high-pressure shock waves in the precipitation solution [9,19]. Though the operation time can be reduced with ultrasound-assisted fractional precipitation, additional devices and energy costs are incurred and the process can be complicated. Therefore, a new fractional precipitation technology that guarantees not only process efficiency but also convenience and feasibility is still needed. Drawing from the cavitation-based fractional precipitation technique, this study developed a new green negative pressure cavitation fractional precipitation technique for efficient purification of paclitaxel from biomass and optimized the key process parameters of negative pressure and operation time. Negative pressure cavitation fractional precipitation is advantageous

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as it provides fast and efficient precipitation with a low investment while utilizing energy efficiently. In addition, the characteristics of the precipitation are quantitatively analyzed through the kinetic analysis of negative pressure cavitation fractional precipitation and the calculation of diffusion coefficients. The study results could be utilized in the eco-friendly commercial mass production of the anti-cancer substance paclitaxel from plant cell cultures.

MATERIALS AND METHODS

1. Sample Preparation for Fractional Precipitation

Suspension cells derived from *Taxus chinensis* were cultured in Modified Gamborg's B5 medium at 24 °C under dark conditions while stirred at 150 rpm [10]. After cell culturing, the plant cells were recovered with a decanter (Westfalia, CA150 Clarifying Decanter) and a high-speed centrifuge (α-Laval, BTPX205GD-35CDEEP). The obtained biomass was mixed with methanol (1 : 1, w/v) and stirred at a room temperature for 30 min. Then methylene chloride was added to the mixture to equal 25% of the extracted solution for liquid-liquid extraction. Paclitaxel was recovered into the lower layer of methylene chloride through phase separation and then concentrated and dried. The dried crude extract was treated with adsorbent (Sylopute) and filtered *in vacuo* at 30 °C and then purified through a silica-gel 60N (Timely, Japan) column (elution: 1.5% (v/v) methanol in dichloromethane). The purified samples (purity: 20.47%) were used in this study.

2. Negative Pressure Cavitation Fractional Precipitation

The schematic diagrams of conventional fractional precipitation without negative pressure and negative pressure cavitation fractional precipitation are presented in Fig. 1. Crude paclitaxel (purity: 20.47%) was dissolved in methanol (pure paclitaxel contents in methanol: 0.5%, w/v), and distilled water was added dropwise while stirring at 335 rpm at room temperature until the methanol to water ratio (v/v) for fractional precipitation of paclitaxel became 61.5 : 38.5. The volume of the batch reactor was 30 mL and the working volume was 15 mL. Negative pressure was imposed into the precipitation solution using a vacuum controller unit (EYELA NVC-3000, Japan) and a diaphragm vacuum pump (EYELA NVP-1000, Japan) to examine the effect of negative pressure. The effect of varying negative pressures (0, −50, −100, −150, and −200 mmHg) at a temperature of 5 °C on the precipitation of paclitaxel was examined. In addition, different operation times (1, 5, 10, and 20 min) were applied to check the precipitation time, yield, and purity of paclitaxel. The precipitate was filtered with filter paper (185 mm, ADVANTEC) at reduced pressure and dried in a vacuum oven (UP-2000, EYELA, Japan) at 40 °C for 24 hr. The contents of paclitaxel in the dried precipitate were analyzed by HPLC.

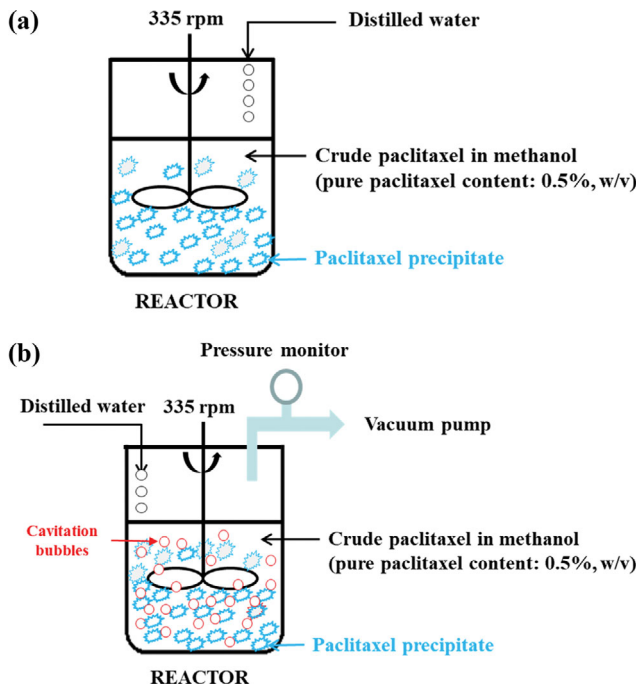

Fig. 1. Schematic diagram of conventional fractional precipitation (a) and negative pressure cavitation fractional precipitation (b) for the purification of paclitaxel.

3. Analysis of Paclitaxel

The paclitaxel contents were analyzed through an HPLC system (SCL-10AVP, Shimadzu, Japan) equipped with a Capcell Pak C18 column (250×4.6 mm, Shiseido, Japan). The gradient elution was performed using a distilled water and acetonitrile mixture (65/35-35/65, v/v) with a flow rate at 1.0 mL/min. The injection volume was 20 μL and the effluent was detected by UV at 227 nm [20]. Authentic paclitaxel (purity: 97%) was procured from Sigma-Aldrich and used as a standard.

4. Measurement of Precipitate Size

The shape and size of the precipitate obtained from the fractional precipitation was measured through an electron microscope (SV-35 Video Microscope System, SomeTech, Korea) [21]. Precipitates were observed under high-magnification (x200) and the size of the precipitate was measured via video images using IT-Plus System (SomeTech, Korea).

5. Estimation of Diffusion Coefficient

The diffusion coefficient (D_{AB}) of the paclitaxel molecule (B) diffused into the fractional precipitation solvent (A) was calculated with Stokes-Einstein equation and can be expressed as Eq. (1) [9,22].

$$D_{AB} = \frac{kT}{61.2\pi r_0 \eta} \tag{1}$$

where, k is the Boltzmann constant (1.38×10^{-23} J/K), r_0 is the diameter of the paclitaxel molecule, η is the dynamic viscosity of the solution, and T is the absolute temperature of the solution. The viscosity of the solution was measured using a viscometer (Viscolite 700, Hydromotion, UK). Each sample was analyzed in triplicate.

6. Kinetic Model

The Johnson-Mehl-Avrami-Kolmogorov (JMAK) equation, which is mainly used in crystallization or precipitation, describes the kinetics of isothermal phase-transformation in the nucleation and particle growth and it is expressed as Eq. (2) [22,23].

$$X = 1 - e^{-kt^n} \tag{2}$$

Eq. (3) can be obtained by linearizing Eq. (2).

$$\log\left(\ln\left(\frac{1}{1-X}\right)\right) = n\log t + \log k \tag{3}$$

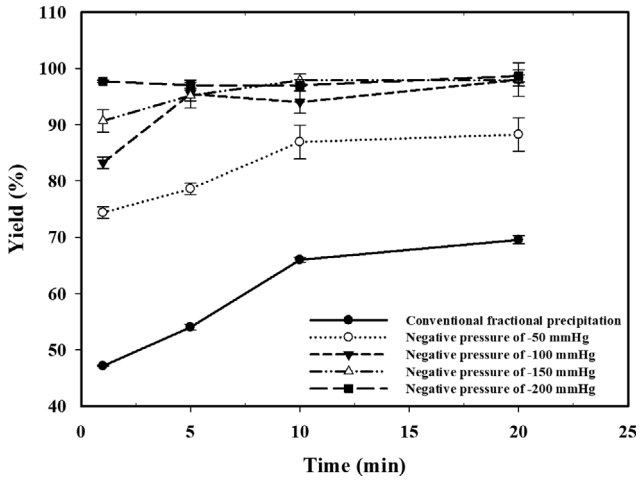

Fig. 2. Effect of atmospheric pressure (0 mmHg) and negative pressures (−50, −100, −150, and −200 mmHg) on the yield of paclitaxel.

where, X is the precipitation fraction, t is the precipitation time, k is the rate constant, and n is the Avrami index, which describes the characteristics of the structure and nucleation of crystals. $\log\left(\ln\left(\frac{1}{1-X}\right)\right)$ vs. logt was plotted using Eq. (3), and n and k were obtained from the slope and intercept of the straight line.

The effectiveness of the JMAK model in the fractional precipitation process of paclitaxel was verified using the coefficient of determination (r^2). The closer the coefficient of determination is to 1, the smaller the error between the experimental value and the calculated value.

RESULTS AND DISCUSSION

1. Development of Negative Pressure Cavitation Fractional Precipitation Process

The results of fractional precipitation with varying negative pressure (0, −50, −100, −150, and −200 mmHg) are presented in Fig. 2. The paclitaxel yields under negative pressures between −50 and −200 mmHg were in the range of 88.2-98.6%. As the negative pressure increased, the paclitaxel yield also increased. Meanwhile, the paclitaxel yield in the conventional precipitation (control) was 69.5% after 20 min of operation time, which was far lower than the fractional precipitations with negative pressure. In addition, the precipitation times for the fractional precipitations under the negative pressures of −50 to −200 mmHg were 1 to 10 min. In particular, when the precipitation was executed at the negative pressure of −200 mmHg, most of the paclitaxel (>97%) could be recovered in a short operation time (1 min). The yield and time for fractional precipitation were remarkably improved compared to the control. The purity of paclitaxel at different negative pressures was in a range of 34-41%, which was negligible (data not shown). This trend was similar to the results of previous studies [10]. The fractional precipitation results of paclitaxel in the previous studies and this study are compared in Table 1. In the fractional precipitation with surface area-increasing materials (glass bead or ion exchange resin) [16,17] and another conventional fractional precipitation without surface area-increasing materials [11,13], the precipitation times were up to 12 hr and up to 72 hr, respectively. The precipitation time in the fractional precipitation where the ratio of distilled water was increased in the precipitation solution (methanol-distilled water mixture) was up to 0.5 hr [18]. Furthermore, the precipitation time in the ultrasound-assisted cavitation fractional precipitation was 0.08-0.50 hr [10]. However, the precipitation time under the negative cavitation precipitation method developed in this study was just 0.02-0.16 hr. That means the application of negative pressure remarkably reduced the operation time of fractional precipitation, which resolved the long operation time issue of conventional fractional precipitation. Such results might be because negative pressure cavitation bubbles themselves acted as sites for heterogeneous nucleation (acting like foreign particles), as was the case in ultrasonic cavitation fractional precipitation, and lowered the free energy barrier for nucleation to promote precipitation [9]. In addition, homogeneous nucleation was promoted due to shock waves and microjets generated by the collapse of negative pressure cavitation bubbles [9,22,24].

2. Kinetic Analysis for the Negative Cavitation Fractional Precipitation

Generally, precipitation particles are created with two stages of nucleation and growth [21]. Therefore, the experimental data (Fig. 2) was applied to the JMAK equation for the kinetic analysis of paclitaxel's precipitation behavior according to the precipitation time. To apply the JMAK equation to the fractional precipitation, $\log\left(\ln\left(\frac{1}{1-X}\right)\right)$ and log t were plotted based on Eq. (2) (data not shown). The Avrami index (n) and rate constant (k) calculated from the slope and intercept of the straight line are presented along with a determination coefficient (r^2) in Table 2. The obtained n val-

Table 1. Comparison of this study and previous studies for fractional precipitation of paclitaxel

Fractional precipitation method	Methanol/water ratio (v/v)	Storage temperature (°C)	Surface area-increasing material	Fractional precipitation time (hr)
Ref. [16]	61.5:38.5	Low temp. (0-4)	Glass beads	~12
Ref. [17]	61.5:38.5	Low temp. (0-4)	Ion exchange resin	6-12
Refs. [11,13]	61.5:38.5	Low temp. (0-4)	No	~72
Ref. [18]	50:50-20:80	Low temp. (0-4)	No	~0.5
Ref. [10]	61.5:38.5	Low temp. (5)	No	0. 08-0.50
This study	61.5:38.5	Low temp. (5)	No	0.02-0.16

Table 2. The values of the kinetic parameters for the fractional precipitation of paclitaxel at different negative pressures

Negative pressure (mmHg)	n (-)	k (min^{-1})	ΔE_a (J/mol) ($E_{a,\ negative\ pressure} - E_{a,\ control}$)	r^2 (-)
Control	0.2166	0.6140	-	0.926
−50	0.1601	1.3183	−1,767	0.890
−100	0.2407	1.8395	−2,537	0.890
−150	0.1738	2.3763	−3,129	0.936
−200	0.0406	3.7120	−4,161	0.828

ues were 0.2166 (control) and 0.0406-0.2407 (at −50 to −200 mmHg). k values (min^{-1}) were 0.6140 (control) and 1.3183-3.7120 (at −50 to −200 mmHg). Though n values in the control were smaller (n: 1.319-1.912) than the conventional fractional precipitation of paclitaxel [9,23], there was no significant difference by the introduction of negative pressure. In the case of the fractional precipitation using negative pressure (−50 to −200 mmHg), k values increased by 2.147 to 6.046 times compared to the control. In addition, k was higher in the negative pressure cavitation fractional precipitation than in the ultrasound-assisted cavitation fractional precipitation (0.9266-1.2311 min^{-1} at an ultrasonic power of 80-250 W) [25]. The activation energies according to negative pressure (−50 to −200 mmHg) using the Arrhenius equation [23] showed ΔE_a values of −1,767 to −4,161 J/mol. The activation energy was therefore reduced by negative pressure during fractional precipitation. The results were similar with those in the ultrasound-assisted cavitation fractional precipitation of paclitaxel [10]. By introducing negative pressure during fractional precipitation, the activation energy (minimum energy required for the reaction) could be reduced, thereby increasing the precipitation rate. Kinetic analysis showed that the JMAK model had a high r^2 value (>0.828), proving that this model was suitable for the fractional precipitation of paclitaxel. For a good statistical model, r^2 should be close to 1.0, and r^2 above 0.75 indicates the aptness of the model [23].

3. Calculation of Diffusion Coefficient

To quantitatively analyze the precipitation characteristics of paclitaxel, the particle sizes, viscosities of the precipitation solutions, and the diffusion coefficients in the conventional fractional precipitation as well as the negative pressure cavitation fractional precipitation were measured and the results are arranged in Table 3. The viscosity of the solution in the control and negative pressure cavitation fractional precipitation were measured as 1.2×10^{-2} and 0.9×10^{-2} g/cm·s. The average particle size in the control was 52.529 μm, while the average particle size in the negative pressure cavitation fractional precipitation was 15.868 μm during 1 to 10 min of precipitation. The average particle size of the precipitate decreased by 3.3 times in the negative pressure cavitation fractional precipitation compared to the control. In general, the reduction of particle size of drug substances has the advantage of improving the dissolution rate, uniformity of drug dispersion, and oral bioavailability during formulation [26]. The diffusion coefficient of paclitaxel D_{AB} was calculated using Stokes-Einstein equation to investigate the diffusion behavior according to the fractional precipitation method (Table 3). While D_{AB} was 6.461×10^{-15} m^2/s in the control, it was 28.532×10^{-15} m^2/s in the negative pressure cavitation fractional precipitation. Therefore, the introduction of negative pressure increased the diffusion coefficient by 4.4 times. Furthermore, D_{AB} was 2.2 times higher in the negative pressure cavitation fractional precipitation (at −200 mmHg) than in the ultrasound-assisted cavitation fractional precipitation (13.212×10^{-15} m^2/s at an ultrasonic power of 250 W) [9]. An increase in the diffusion coefficient significantly affects homogeneous nucleation in fractional precipitation [22,27,28]. This increase in diffusion coefficient is considered to be due to the cavitational phenomena caused by the collapse of negative pressure cavitation bubbles [9,22,29].

CONCLUSIONS

Fractional precipitation using negative pressure was developed to remarkably improve the fractional precipitation efficiency of paclitaxel. While the paclitaxel yield without negative pressure (control) was 69.5%, it was 88.2 to 98.6% through the negative pressure cavitation fractional precipitations at −50 to −200 mmHg, proving that precipitation efficiency was improved by the introduction of negative pressure. In particular, when the fractional precipitation was executed under the negative pressure of −200 mmHg, most of the paclitaxel could be recovered (>97%) in a short operation time (1 min). A kinetic study by applying the experimental data to the JMAK equation showed that the precipitation rate constant increased by 2.147 to 6.046 times at negative pressures of −50 to −200 mmHg when compared with the control. In addition, the changes of activation energy in the negative pressure cavitation

Table 3. Effect of negative pressure cavitation bubbles on the mean particle size and diffusivity

Fractional precipitation type	Mean particle size (μm)	Viscosity ($\times10^2$ g/cm·s)	Diffusion coefficient ($\times10^{-15}$ m^2/s)
Control*	52.529	1.2	6.461
With negative pressure cavitation bubbles (−200 mmHg)	15.868	0.9	28.532

*Without negative pressure (atmospheric pressure, 760 mmHg)

fractional precipitation were −1,767 to −4,161 J/mol at −50 to −200 mmHg compared to the control. By applying negative pressure during fractional precipitation, the activation energy was reduced, which increased the precipitation rate. Furthermore, the average particle size of the precipitate was reduced by 3.3 times in the negative pressure cavitation fractional precipitation compared to the control, and the diffusion coefficient of paclitaxel increased by 4.4 times. Such an increase to the diffusion coefficient ultimately improved the precipitation efficiency by promoting homogeneous nucleation in the factional precipitation.

ACKNOWLEDGEMENTS

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Government of Korea (MSIT) (Grant Number: 2021R1A2C1003186).

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