Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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Online publication date: 23 April 2002

To cite this Article Zhang, Zhiqiang and Su, Zhiguo(2002) 'Catalysis mechanism to increase taxol from the extract of *Taxus cuspidate* callus cultures with alumina chromatography', Separation Science and Technology, 37: 3, 733 — 743
To link to this Article: DOI: 10.1081/SS-120001457
URL: http://dx.doi.org/10.1081/SS-120001457

SEPARATION SCIENCE AND TECHNOLOGY, 37(3), 733–743 (2002)

CATALYSIS MECHANISM TO INCREASE TAXOL FROM THE EXTRACT OF *TAXUS CUSPIDATE* CALLUS CULTURES WITH ALUMINA CHROMATOGRAPHY

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ABSTRACT

In our previous research, we had reported a chromatographic protocol using basic Al_2O_3 column to separate and recover taxol from the extract of *Taxus cuspidate* callus cultures. On the basis of the amount of taxol detected in the starting materials of the crude plant extract, the recovery of taxol was more than 170% after the basic Al_2O_3 column chromatography step. It was found that the main source of taxol increase was 7-epi-taxol in the crude extract conversion to taxol by catalysis of the Al_2O_3 medium. In this paper, the catalysis mechanism of Al_2O_3 in converting 7-epi-taxol to taxol was developed by investigating the surface structure of alumina and the formation of activity core of catalysis. It was found that the type of Al_2O_3, the content of Al_2O_3 in the stationary phase, and the concentration of H_2O in the mobile phase of chromatography could influence the isomeric reaction. The isomerization of 7-epi-taxol to taxol was under the influence of Lewis souci and basic activity

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cores on the surface of alumina. The strength and type of activity core controlled the procedure of reaction.

Key Words: Alumina; Catalysis; 7-Epi-taxol; Mechanism; Taxol

INTRODUCTION

Taxol is a new kind of efficient anti-cancer drug, first obtained from *Taxus* spp (1). Currently, the main commercial sources of taxol are the bark of *Taxus* spp and tissue culture, but *Taxus* spp and the tissue callus grow slowly and yield relatively low amounts of taxol. Furthermore, the content of taxol is very low in plant and callus cultures with large amounts of impurities. The supply of taxol is limited, and it is also difficult to obtain a high recovery with high purity of taxol in various processes.

In order to solve these problems, an efficient method is to find various procedures to increase the yield of taxanes from *Taxus* extract. Much effort has been devoted to studying taxol separation and purification (2–4). The *Taxus* extract contains some combined taxanes, including sugar bound taxols, such as 7-xylosyl taxol, isolated and identified in the literature (5). Durzan et al. (6) used xylanase to free taxol from the taxane-bound compounds. Carver et al. (7) indicated an increase in the taxol recovery from the extract of *Taxus brevifolia* with ion-exchange solvent treatment. In our previous research, we had reported a chromatographic protocol using Al_2O_3 phase to separate and recover taxol from the extract of *Taxus cuspidate* callus cultures (8). Significant results were obtained with the basic Al_2O_3 column chromatography, where the recovery was more than 170% with a purity increase from 0.65 to 29.4%. The main source of taxol increase was the isomerization of 7-epi-taxol to taxol catalyzed by alumina, and the taxol could also be decomposed to baccatin III and 10-deacetylbaccatin III in the Al_2O_3 column.

In this paper, the surface structure, formation of the activity core of basic alumina, and the process of basic Al_2O_3 catalysis were investigated. The catalysis mechanism of converting 7-epi-taxol to taxol from the extract of *Taxus cuspidate* callus cultures was developed.

MATERIALS AND METHODS

Materials

Taxol was obtained from Sigma (St. Louis, MO). Acetonitrile, methanol (HPLC-reagent grade), and chloroform (analytical grade) were purchased from

Beijing Chemical Reagent Company. Water was deionized and double distilled. Rotary evaporator was from Kelong Company (Beijing, China). Silica gel (50–70 μm) was supplied by Qindao Ocean Chemical Works (Shandong, China). Alumina medium (50–70 μm) was supplied by Shanghai Chemical Works (Shanghai, China). The high-pressure liquid chromatography (HPLC) system was Beckman FL-750. C_{18} HPLC column (4.5 mm × 250 mm) also was purchased from Beckman Instruments Inc. (CA).

Methods

Sample Preparation

Callus cultures were established from *Taxus cuspidate* as described by Xu et al. (9). The six to eight week old, slow-growing callus samples were collected, freeze-dried, and powdered, then methanol was added to the extract at an ambient temperature for 2 days. The resulting solution was filtered through filter paper, and the methanol solution was evaporated to dryness on a rotary evaporator at 40–50°C. The residue was redissolved in chloroform for the subsequent isolation steps.

Preparation of the Silica–Alumina Medium

In this paper, silica–alumina refers to the adsorption chromatographic support containing silica gel and alumina. The silica gel and alumina were mixed in a ratio ranging from 0:100 to 100:0, and conducted at 650°C for 2 hr, then cooled in a dryer to room temperature for the next chromatographic step.

Determination of Total Acidity, Lewis Acidity, and Bronsted Acidity

Determination of total acidity, Lewis acidity, and Bronsted acidity of the silica–alumina medium was performed according to Tanabe's method (10).

Al_2O_3 Chromatography and the Conversion of 7-Epi-taxol to Taxol

The basic Al_2O_3 with particle size 50–70 μm was loaded onto the column (15 mm × 250 mm), and washed with chloroform. After the column was equilibrated with chloroform/methanol (99:1, v/v) at the rate of 2.0 mL/min, 200 mg of the sample mixture dissolved in chloroform/methanol (99:1, v/v) was

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loaded, the column was then closed, and the sample was kept on the column for several minutes. Isocratic elution was performed with chloroform/methanol (95:5, v/v) at 3.5 mL/min. Fractions of the elution were evaporated to dryness on a rotary evaporator at 40–50°C. The residue was resuspended in methanol for HPLC analysis.

High Pressure Liquid Chromatography Analysis

Samples analysis was carried out with HPLC (FL-750, Beckman Instruments Inc., CA) using a C_{18}-silica column (4.5 mm × 250 mm, Beckman Instruments Inc., CA). A 20 μL sample was injected, and the elution was monitored at 227 nm. The mobile phase for isocratic elution was methanol/acetonitrile/water (25:35:40, by volume). The elution rate was 1.0 mL/min. Taxol was quantitated by comparing the average peak response of the sample to that of the standard (Sigma, St. Louis, MO). A linear relationship ($r^2 = 0.9996$) between the peak area and the amount of taxol was shown over the range 0.04–0.96 mg/mL.

RESULTS AND DISCUSSION

Silica Gel and Basic Al_2O_3 Adsorption Chromatography to Separate Taxol

The results of silica gel and basic Al_2O_3 adsorption chromatography to separate taxol from the extract of *Taxus cuspidate* callus cultures are shown in Table 1. The basic Al_2O_3 adsorption chromatography was more efficient than the silica gel adsorption chromatography for initial separation of taxol. The amount of taxol increased, as shown by the recovery of more than 170% after the basic Al_2O_3 chromatography step, with purity increase from 0.65 to 29.4%. In contrast, the silica chromatography only gave 99% recovery with purity increase from 0.65 to 15.3%.

To inspect the recovery, the spiked sample containing definite amounts of the standard taxol was analyzed using HPLC, and the recovery was obtained by comparing the peak response of taxol in the sample and the spiked sample. Thus, the recovery of 170% was not due to erroneous taxol analysis, but the main source of taxol increase was the isomerization of 7-epi-taxol to taxol (8). There should be some activity cores existing on the surface of basic alumina medium, which could catalyze the conversion of other taxanes to taxol.

Table 1. The Comparison of Silica-Gel and Basic Alumina Chromatography to Separate Taxol (Total *W*: Total Weight of Sample)

Method	Total *W* (mg)	Taxol (mg)	Purity (%)	Recovery (%)
Crude sample	5000	32.5	0.65	100
Silica-gel chromatography	210.5	32.2	15.3	99.0
Al_2O_3 chromatography	195.6	55.1	29.4	175.1

Formation of Activity Core on the Surface of Alumina

Figure 1 shows the formation of activity core on the surface of alumina. A high activity of alumina as the chromatographic medium was obtained after being dehydrated. The alumina surface also contains some activity cores apart from basic (O^{-1} and O^{-2}), Lewis acidic (L acidic core) and Bronsted acidic cores (B acidic core). The L acidic core could convert to the B acidic core on adsorption of H_2O by the alumina medium. This structure gives the adsorption and catalysis capabilities to alumina.

Influence of Alumina Properties on the Separation of Taxol

Figure 2 shows separation of taxol on basic (A), neutral (B), and acidic (C) alumina adsorption columns. Among the three kinds of alumina adsorption

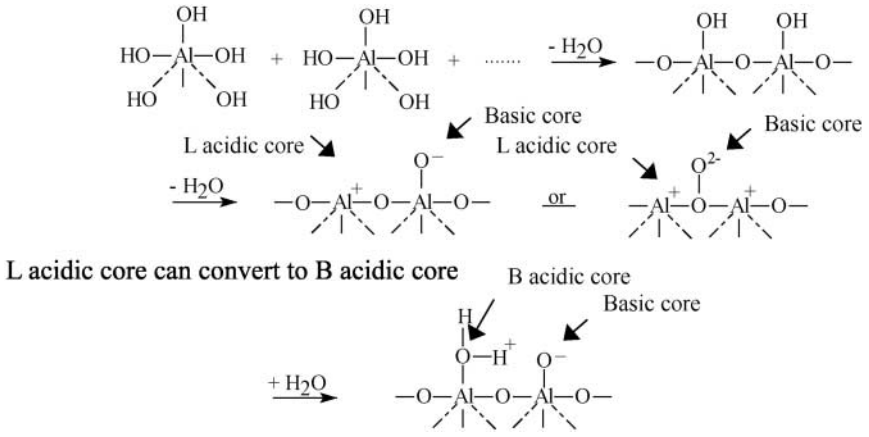

Figure 1. The formation of acidic core and basic core on the surface of alumina.

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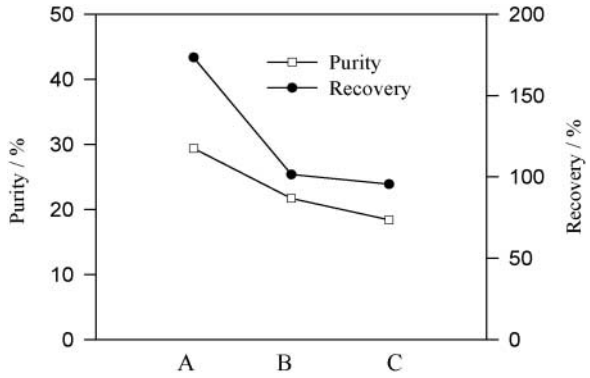

Figure 2. The influence of acidity or basicity of Al_2O_3 on the catalysis activity. Experimental conditions: the initial feed: extract of *Taxus*, feed loading: 200 mg, column: 15 mm × 260 mm, washing with $CH_3OH/CHCl_3$ (1:99, v/v), elution with $CH_3OH/CHCl_3$ (4:96, v/v) A: basic alumina, B: neutral alumina, and C: acidic alumina.

chromatography, the basic alumina chromatography was the most efficient in increasing the content of taxol. After the basic alumina chromatography step, not only the recovery was more than 170%, but also the purity of taxol increased from 0.65 to 21.7%. In contrast, the neutral alumina chromatography gave 101.5% recovery with a purity increase from 0.65 to 15.3%. The acidic alumina chromatography only gave 95.6% recovery with a purity increase from 0.65 to 18.4%.

While analyzing the alumina medium preparation, it was found that the neutral and acidic alumina media were obtained from the basic alumina by thorough washing of the acidic solution, and the basic core reacting with the acid solution to become either a neutral or an acidic core. So, on the surface of neutral or acidic alumina medium, there was hardly any basic core. However, the results from Fig. 1 show that without the basic core on the surface of alumina medium, the isomeric reaction of the conversion of 7-epi-taxol to taxol could not be conducted. In brief, for the isomerization of 7-epi-taxol to taxol, the basic activity core is essential.

Influence of H_2O in the Mobile Phase on Separation of Taxol

Figure 3 shows the influence of H_2O content, in the mobile phase, on the recovery in basic alumina chromatography. Addition of H_2O in the mobile phase decreased the recovery of taxol was greatly from more than 170 to 100%, and the purity of taxol was minimum at the 0.07% of H_2O in the mobile phase. Further increase in H_2O content up to 0.1% showed an increase in the trend of purity.

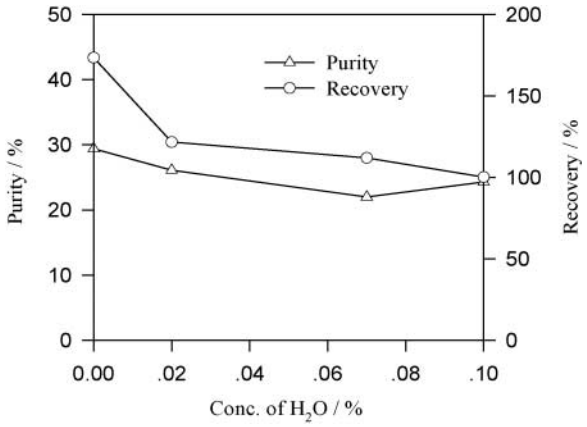

Figure 3. The influence of the content of H_2O in the mobile phase on the catalysis activity. Experimental conditions: the initial feed: extract of *Taxus*, feed loading: 200 mg, basic Al_2O_3 column: 15 mm × 260 mm.

According to Synder et al. (11), a little amount of H_2O in the mobile phase could remove the strongest adsorption centers of the medium, the presence of H_2O could improve the separation of adsorption chromatography, and obtain a higher recovery and purity of products. However, the experimental results did not show the same trends. Especially, when the H_2O content in the mobile phase was up to 0.1%, there was no 7-epi-taxol conversion to taxol as shown by the about 100% recovery after the basic alumina adsorption chromatography step. It was possible that the presence of water denatured some activity centers of reaction at the surface of alumina, the quantity of taxol converting from taxanes decreased, alternatively, the recovery of taxol was lower.

From the formation of the activity cores on the surface of alumina shown in Fig. 1, it was known that the L acidic core could convert to B acidic core with the addition of H_2O. As the amount of L acidic core decreased, the capability of alumina to catalyze 7-epi-taxol to taxol also decreased. Therefore, L acidic core is also essential for this isomeric reaction.

Mixture of Silica and Alumina Medium to Catalyze and Separate Taxol

The content of basic alumina in silica–alumina support ranged from 0 to 100%. Figure 4 shows the comparative results of these media with different contents of basic alumina to separate and recover taxol from the extract of *Taxus*

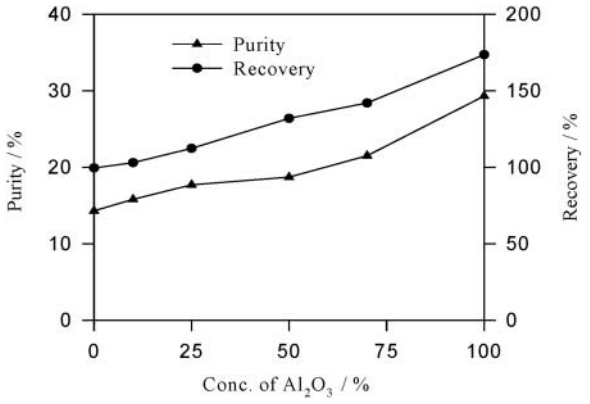

Figure 4. The catalysis and separation of taxol with silica and Al_2O_3 gel. Experimental conditions: the initial feed: extract of *Taxus*, feed loading: 200 mg, column: 15 mm × 260 mm, washing with $CH_3OH/CHCl_3$ (1:99, v/v), elution with $CH_3OH/CHCl_3$ (4:96, v/v).

cuspidate callus cultures. When there was no basic alumina present in the mixture medium, the recovery of taxol could only reach about 99%, with purity increase from 0.65 to 15.3%. With addition of basic alumina to the mixture medium, both the recovery and purity of taxol increased. Especially, the recovery was more than 100%, which indicated that the conversion of other taxanes to taxols increased the amount of taxol in the feed. The increase in the recovery and purity of taxol was maximum when the content of basic alumina was 100%. Evidently, the concentration of basic alumina in the mixture medium had strong influence on the isomeric reaction of the conversion of 7-epi-taxol to taxol.

Relationship of the Strength of Acidic Core with the Content of Alumina

Figure 5 shows the relationship of the strength of acidic cores of silica–alumina medium with the content of alumina. With an increase in the concentration of alumina in the mixture, the total acidity and Lewis acidity increased. Contrarily, an increase in the Bronsted acidity was maximum at 30% (G/G) concentration of alumina.

The comparison of Figs. 4 and 5 indicates that the more stronger the Lewis and the total acidity were, the higher was the recovery of taxol, and the recovery of taxol did not depend on the Bronsted acidity. Clearly, the isomerization of 7-epi-taxol to taxol was under the influence of the Lewis souci and the basic

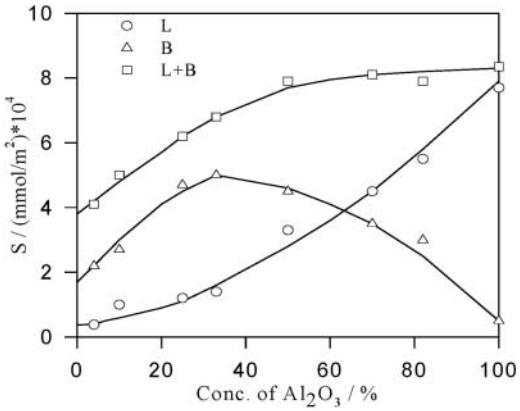

Figure 5. Relationship of the acidity to the concentration of alumina in the silica–alumina.

activity cores on the surface of alumina. The strength and type of activity core controlled the procedure of the reaction.

Possible Mechanism

Figure 6 shows the structures of taxol and 7-epi-taxol (12). It is seen that the small difference between taxol and 7-epi-taxol was due to the different position of –H and –OH at C-7 in the structure. The activation of C–H is enough to cause the isomeric conversion of 7-epi-taxol to taxol. The basic alumina has the capability to induce this process at room temperature (13).

In conclusion, the possible mechanism of 7-epi-taxol conversion to taxol on the surface of basic alumina can be described with positive carbon ion procedure.

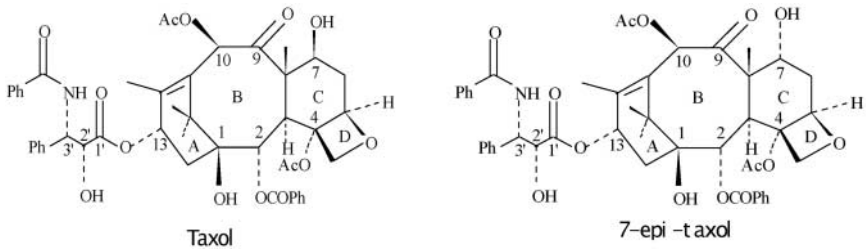

Figure 6. The structures of taxol and 7-epi-taxol.

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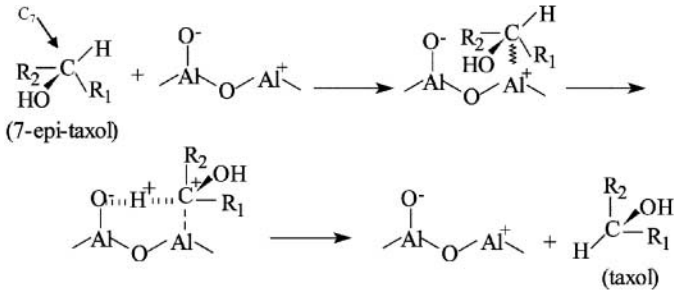

Figure 7. The isomeric mechanism of 7-epi-taxol on the surface of alumina.

The primary condition is that the medium should have the capability of both adsorption and catalysis. Figure 7 shows the steps of this isomeric reaction. First, 7-epi-taxol is attracted and adsorbed by the adsorption cores of basic alumina. Secondly, the L acidic cores attack the C-7 atom from the back of –OH group, while the basic cores compete with C-7 atom for the H^+, and induce the reaction as nucleophilic substitution (S_N2 reaction). Thirdly, the groups on the C-7 atom are exchanged and rearranged, and the structure of 7-epi-taxol is changed. An unstable positive carbon ion is formed after this molecular transposition. Finally, the positive carbon ion de-adsorbs from the L acidic core, and competes with the basic core for the H^+, and forms the new structure of taxol.

Therefore, the isomerization of 7-epi-taxol to taxol was under the influence of the Lewis souci and the basic activity cores on the surface of alumina. The strength and type of activity core controlled the procedure of the reaction. Catalyzed by alumina, the reaction could occur at room temperature also.

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Received June 2000
Revised April 2001

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