Purification of Epothilones A and B with Column Chromatography on a Sephadex LH-20 checked 18/5 Abstract. A simplified and efficient method was developed for the large-scale purification of the epothilone A and B from fermentation products of Sorangium cellulosum after organic solvent extraction in this paper. Extractant from XAD-16 resin with tetrachloromethane containing epothilones was concentrated under vacuum, subjected to Sephadex LH-20 column chromatography, and eluted with several solvents. Fractions containing epothilones are pooled, concentrated, and applied to a second cycle of column chromatography with other solvents. Result showed that elution with acetone gave the best purity of epothilones [78.1% by high-performance liquid chromatography (HPLC) than that with pure methanol, pure ethanol, 70% (v/v) aqueous methanol or methanol-dichloromethane (1:1, v/v).] Then, when flow rate was 0.2 mL/min and sample amount (epoA) was 1 mg in second cycle, elution with methanol was resulted in complete separation between epothilone A and B, and further improved separately the purity of epothilone A and B to 90.27% and 77.34%. This simplified purification scheme avoided the loss of expensive epothilones in the common silica gel separation process and achieved the separation of epothilone A and B, significantly reduce the cost of the production without preparative liquid chromatography, or other equipment. Introduction	Tinh chế Epothilones A và B bằng phương pháp sắc ký cột trên Sephadex LH-20 Tóm tắt. Trong nghiên cứu này chúng tôi xây dựng một phương pháp đơn giản và hiệu quả để tinh chế quy mô lớn epothilone A và B từ các sản phẩm lên men của Sorangium cellulosum sau khi chiết tách bằng dung môi hữu cơ. Chất chiết tách từ nhựa XAD -16 cùng với tetrachloromethane chứa epothilones được cô đặc trong chân không, trải qua quá trình sắc kí cột Sephadex LH-20, và tách rửa bằng một số dung môi. Các đoạn chứa epothilones được gộp lại, cô đặc, và trải qua chu trình sắc ký cột thứ hai với một số dung môi khác. Kết quả cho thấy rằng rửa giải với acetone làm cho epothilones có độ tinh khiết tốt nhất[78.1% với phương pháp sắc ký lỏng hiệu suất cao (HPLC) so với methanol tinh khiết , ethanol tinh khiết, 70% (v / v) methanol lỏng hoặc methanol-dichloromethane (1:1 , v / v) .] Sau đó, khi tốc độ dòng chảy bằng 0,2 ml / phút và lượng mẫu (epoA) là 1 mg trong chu kỳ thứ hai , rửa giải với methanol làm cho epothilone A và B tách hoàn toàn với nhau, và tiếp tục cải thiện độ tinh khiết của epothilone A và B đến 90,27 % và 77,34 % . Phương pháp tinh chế đơn giản hóa này tránh được sự tổn hao epothilones trong quá trình tách gel silica thông thường và có thể tách được epothilone A và B, làm giảm đáng kể chi phí sản xuất mà không cần sắc ký lỏng điều chế, hoặc các thiết bị khác .

Epothilones are a type of macrolide produced by Sorangium cellulosum, which were first described by G. Hofle et al. in 1993 (Fig. 1) [1-2]. They have become an important drug-mining resource for their Taxol®-like microtubule stabilization [3-4]. Epothilones are synthesized by Sorangium cellulosum, and the main components are epothilone A and B.

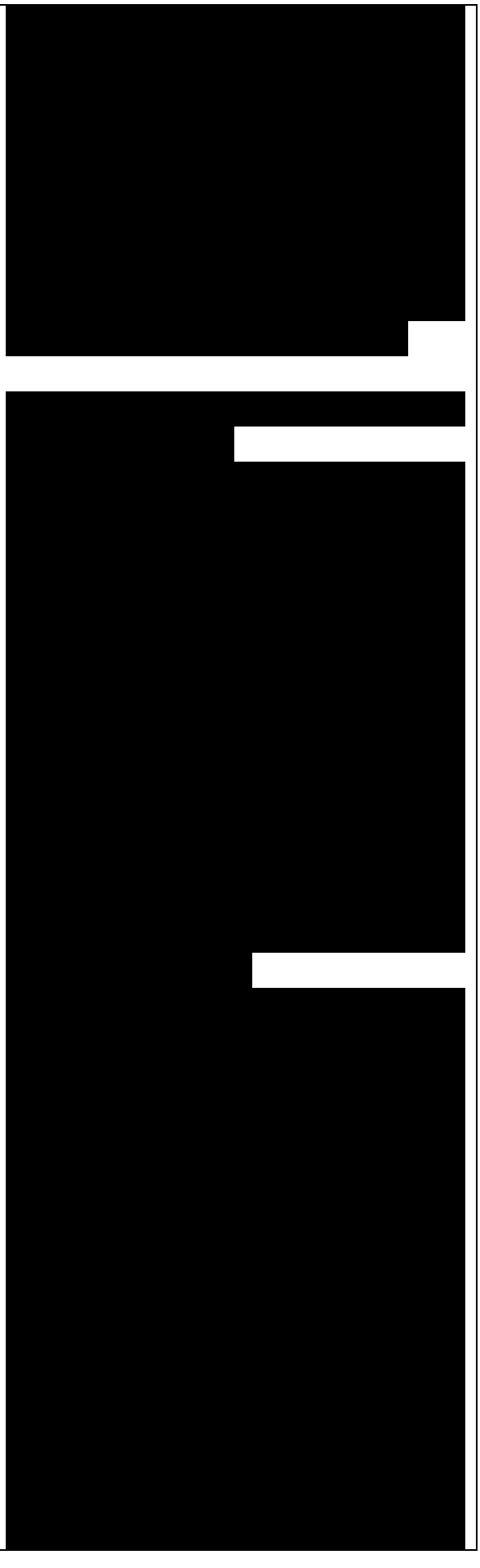


Fig. 1 Chemical structure of Epothilone A and B

Epothilones have been of increasing interests for its anticarcinogenic, which are found to kill dividing cells by stabilizing microtubules at nanomolar and subnanomolar concentrations [5]. In 2007, the analog of epothilones, ixabepilone was developed by Bristol-Myers Squibb Pharmaceutical Research Institute and was approved the listing by FDA, which was mainly used for the treatment of metastatic, prostate cancer and locally advanced breast cancer [6-9].

Although epothilones have been used for their great potential cancer treatment, but the inefficiency in isolation and purification is still one of the main limiting factors that restricting industrialization process of epothilones outside the lower productivity [10]. Traditionally, preparative HPLC and silica gel column chromatography were the most common methods for separation. Nevertheless, expensive cost and low-throughput of preparative HPLC caused it difficult to be applied in industrial production. Besides, low rate in

recovery of product by the silica gel column chromatography was also unacceptable for the cost control.

Sephadex gel chromatography is a kind of filtration chromatography. It depends on the principle that the outflow speed of substance is proportional to the size of molecular. The expansion degrees of sephadex gel are various in different solvents, which lead to diverse effects of separation. As reported, sephadex gel chromatography was only applied for the preliminary purification of epothilone A and B.

In this study, various solvents single or combined and other parameters of Sephadex LH-20 column chromatography were optimized for the further isolation and purification of epothilone A and B, intending to prepare pure epothilone A and B in large-scale.

Material and Methods

Preparation of Epothilones Extract.

Sorangium cellulosum So2161 was provided by School of Life Sciences, Shandong University. So2161 was cultured in ferment medium which contain 1.5% sterilized XAD-16 resin. After fermentation, the dried resins were soaked with tetrachloromethane at normal temperatures and pressures. The solid-liquid ratio is 1:4 (w/v). After 4 hours, the extract was obtained by filtration with qualitative filter paper. Residues were extracted under the same conditions for three times. All extracts were combined, evaporated under vacuum at 45°C to approximately 10 mL. Then, the

samples were stored at 4°C before using it. Microfiltration through 0.45-p.m membrane (Genosys Tech-Trading Co., Ltd, Beijing, China) or centrifugation (10,000 r/min and 10 mins) was performed before column chromatography purification (Fig. 2).

Oollected fractions containing epothilones Concentrated

Fig. 2 Separation procedure of target compounds from crude sample

Sephadex LH-20 Column Chromatography Separation Procedure. Column chromatography was performed with Sephadex LH-20, which was got from Amersham Pharmacia Biotech (AB, Uppsala, Sweden). About 200 g Sephadex LH-20 was swelled in 1000 mL of pure methanol at room temperature for 24 h. Then, the Sephadex LH-20 was deaerated and poured into glass column (130^1.5 cm). The process of equilibration needed approximately two column volumes of mobile phase.

Partial impurities were removed by extracting with tetrachloromethane. In consideration of the dissolving properties of epothilones and frequently-used solvent in Sephadex LH-20 column chromatography, we selected methanol, ethanol, acetone, methanol-water (7:3, v/v) and methanol -dichloromethane (1:1, v/v) as eluent. Better purity grades and degree of separation between epothilone A and epothilone B were proposed as the assessment standard for this study. Under the following conditions: elution flow rate, 0.6mL/min; loading quantity of sample, epothilone A=6.0mg; column temperature, room

temperature.

Analytical Methods. The concentrations of Epothilone A and B were determined using a high-performance liquid chromatography (HPLC) system (Shimadzu, Japan), equipped with two LC-20AT pumps and an SPD-20A UV-Vis detector, a 5^m RP-C18column (4.1*250 mm; Agilent, USA) at 35°C. The mobile phase was composed of methanol-water (70:30 v/v). The flow rate was kept at 1.0 mL/min and UV detection was set at 249 nm. The injection volume was 10 |iL.

Fig. 3 The purity of epothilones subjected to Sephadex LH-20 column chromatography with different solvents.

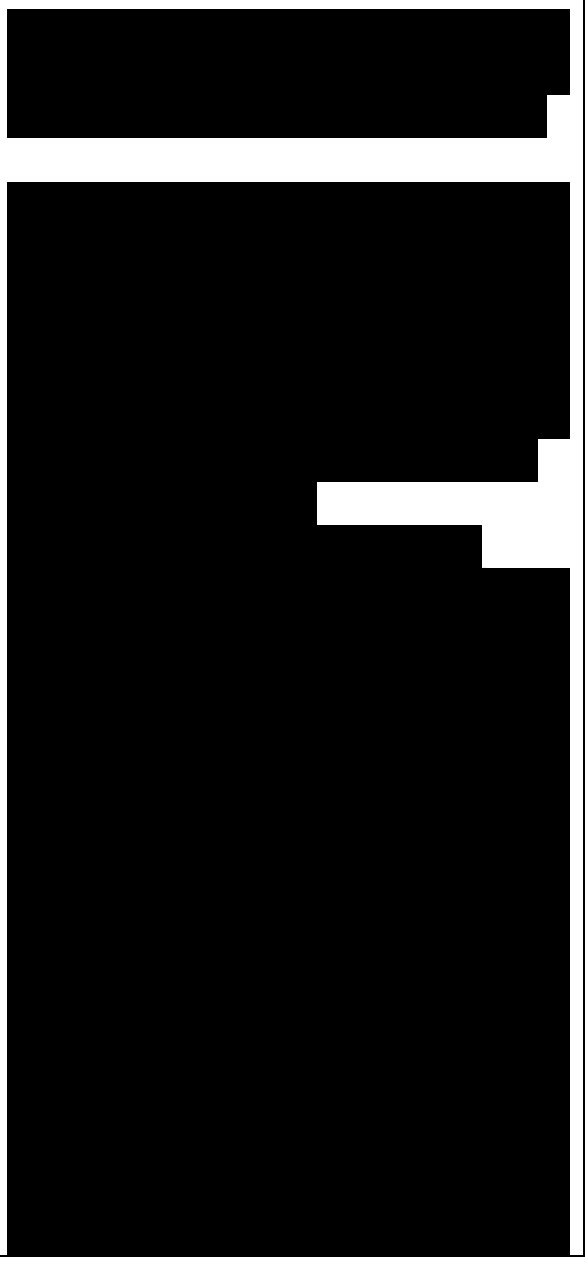


Fig. 4 The outflow trend of epothilones with different solvent in Sephadex LH-20 column chromatography. A: methanol-water (7:3, v/v); B: ethanol; C: methanol; D: acetone; E: methanol-dichloromethane (1:1, v/v).

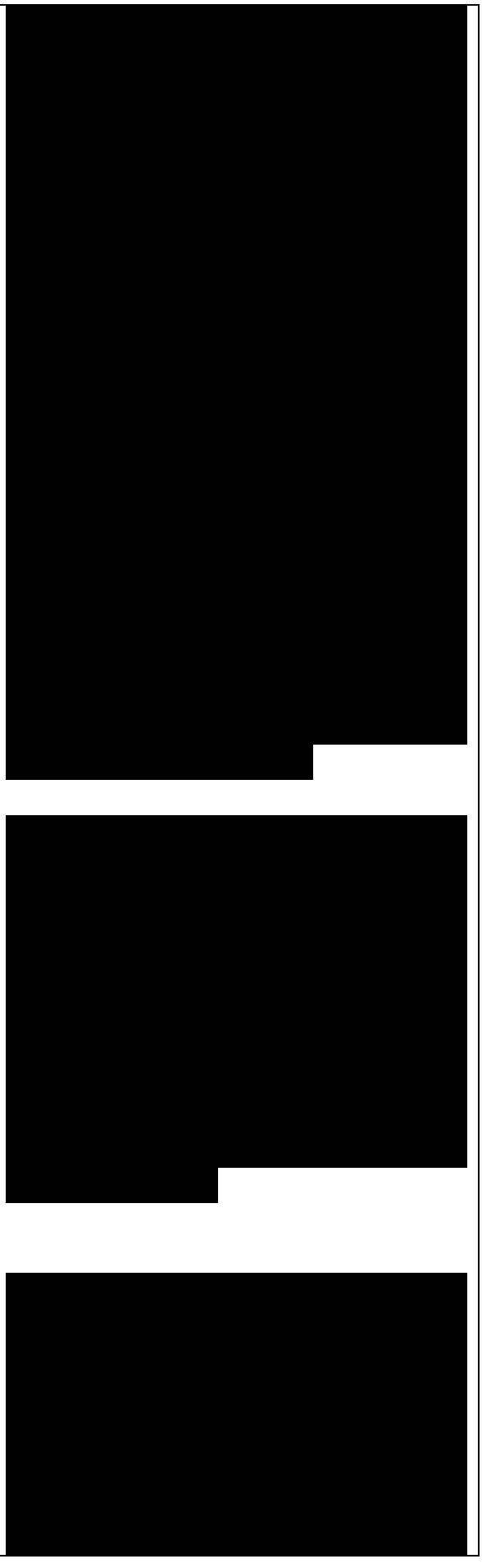
Results and Discussion

Comparing with Different Solvents. Resulted showed that the purity of epothilones could reach 78.1% (HPLC analysis) when acetone was used as the mobile phase, which was much more than methanol (47.4%), ethanol (36.1%), methanol-dichloromethane (1:1, v/v) (49.3%) and methanol- water (7:3, v/v) (55.6%) (Fig. 3). Furthermore, Sephadex LH-20 gels have different swelling ratios in different solvents. The swelling ratio of Sephadex LH-20 gel was approximately 2.5 (mL gel/ g dry gel) in acetone, which was less than the others (between 3.8 and 4.4). Therefore, we thought that the

pore volume of Sephadex LH-20 gel in acetone might be beneficial to improve the purity of epothilones. Meanwhile, we found a best resolution (0.37) between epothilone A and B was obtained when methanol was used as the mobile phase (Fig. 4), better than the others (all below 0.26). The results showed that the relationship between swelling ratios of Sephadex LH-20 gels and the resolutions were not close connected. Prior research has shown that the polarity of eluent played an important role for the purification of polar substance in Sephadex LH-20 gel column chromatography. We could speculated that the polar of methanol might be most suited for increasing the resolution between epothilone A and epothilone B in Sephadex LH-20 column chromatography.

Comparing with Different Flow Rates. The flow rate was then optimized. The sample, with most of impurities removed by column chromatogram with acetone, was obtained to be used for the second cycle of elution. Methanol was chosen as solvent to the benefit of baseline separated between epothilone A and epothilone B. Results showed that baseline separated was got at a flow rate of 0.2 mL/min (Fig. 5.a).

Comparing with Different Loading Quantity of Sample. Furthermore, the improvement of resolution benefits the reduction of loading quantity of sample in the gel column chromatography. The sample was pretreated by column chromatogram with acetone. The loading quantity of



sample was gradually reduced with methanol as the mobile phase in the gel column chromatography. Results showed that epothilone A and B were completely separated from each other when the loading quantity of sample (epoA) was 1.0 mg (Fig. 5.b).

Validation of the Optimized Condition. Based on the above experimental result, we obtained a process design of epothilone A and B purification by Sephadex LH-20 column chromatography. Firstly, the prepared sample was purified by Sephadex LH-20 column with acetone followed the condition: elution flow rate, 0.6mL/min; loading quantity, epothilone A=6.0mg; column temperature, room temperature. The fractions containing epothilones were collected and concentrated to elution as the sample for the further column chromatography with condition: methanol as eluent, flow rate, 0.2 mL/min, loading quantity (epoA) 1.0 mg. The purity of epothilone A was 90.27%, and B reached 77.34% (Fig. 6).

Recovery of Epothilone A and B by Sephadex LH-20 Column Separation. After being calculated, the recovery of epothilone A was 98.2±1.8%, and B was 93.7±2.2%. The loss of epothilones was acceptable during the purification process.

Fig. 5 The effect of flow rate and loading quantity on the resolution between epothilone A and B.

Fig. 6 HPLC chromatogram of purified epothilone A and B. A Symmetry C18 column was used as the stationary phase. Detecting

conditions: The solvent: methanol-water (7:3, v/v); The flow rate: 1.0 mL/min; The absorbance: 249nm.

Conclusion

Epothilones have good application prospects and market value because of its anticancer activity [11-13]. This study demonstrated that clean separation between epothilone A and B could be realized through a combination of solvents. Meanwhile the purities and recoveries of epothilone A and B isolated following the above method were acceptable. Compared with preparative high performance liquid chromatography (prep-HPLC) and high-speed counter-current chromatography (HSCCC) methods, the established method had the advantages of simple equipment and low cost. The results of this study might be benefit for developing a simple and efficient method for the large-scale purification of the epothilone A and B.