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聚合物纳米微球分离纯化放线菌素 D 的研究

杨煌建,张祝兰*,王德森,任林英,唐文力
福建省微生物研究所,福州 350007

摘　要:本实验考察从链霉菌发酵产生的放线菌素 D 粗提物中分离制备高纯度放线菌素 D 的工艺,以聚合物纳米微球为固相载体,对聚合物纳米微球型号、洗脱条件进行优化。最终确立放线菌素 D 的分离纯化工艺为:采用 PS40-300(以聚苯乙烯/二乙烯基苯聚合物为基质,粒径 40 μm,孔径 300Å)纳米微球作为层析填料;以链霉菌发酵产生的放线菌素 D 粗提物为上样样品,用适量 95% 乙醇(V/V)充分溶解,上样量为 0.5 g/100 mL,控制洗脱速度为 5.0 mL/min,以 65% 乙醇水(V/V)洗脱,收集纯度 95% 以上的洗脱液,真空浓缩得到高纯度的放线菌素 D。结果表明该分离纯化工艺,纯度与收率都较高,流程简单、可行,为该产品产业化开发奠定基础。

关键词:聚合物纳米微球;分离纯化;放线菌素 D
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Separation and Purification of Actinomycin D by Nanoscale Polymer Particles

YANG Huang-jian, ZHANG Zhu-lan*, WANG De-sen, REN Lin-ying, TANG Wen-li
Fujian Institute of Microbiology, Fuzhou 350007, China

Abstract: In this study, the separation and purification process of high-purity actinomycin D was investigated. The types of nanoscale polymer particles and the elution conditions were observed. The optimized processes were as follows: PS 40-300 (based on polystyrene/divinylbenzene mediaparticle size 40 μm, pore size 300Å) nanoscale polymer particles was determined as the optimal chromatographic filler. Actinomycin D crude samples were extracted from the fermentation broth of *Streptomyces* strain. The crude extracts were dissolved in the ethanol-water (95∶5, V/V) and loaded to the column wet-packed with polymer particles. The amount of sample was 0.5 g/100 mL filter, the elution speed was 5.0 mL/min, and ethanol-water (65∶35, V/V) was used as the eluent. Actinomycin D with high purity and recovery were obtained under the optimal conditions. The established process was simple and feasible for further industrialization of actinomycin D.

Key words: nanoscale polymer particles; separation; actinomycin D

Introduction

Actinomycin D (AMD, Fig. 1A) belongs to the group of antibiotics containing two pentapeptide lactone rings at positions 1 and 9 of the chromophore phenoxazinone ring isolated from various strains of *Streptomyces* and related species[1-3]. It has been used clinically as a chemotherapeutic agent, with excellent results in the treatment of Wilms' tumor and gestational choriocarcinoma[4-6]. In the fermentation of AMD, the producing strain also produces one major minor component actinomycin S3 (AMS, Fig. 1B)[7]. The similar chemical structures among these two products make it very difficult to separate them from each other, which necessitate an efficient method for solving this problem.

The isolation of AMD has been, so far, based on normal chromatography, and the silica or acidic aluminum oxide was used as media. But the separation procedure of this technique is not efficient to obtain AMD[7]. Nanoscale polymer particle is the high performance polymeric reversed phase chromatography media. The truly spherical and monodisperse sorbent with optimized pore structure provides excellent selectivity, low back pressure, high retention capacity and recovery yield, minimal elution volume, exceptional batch-to-batch reproducibility, with outstanding chemical and pH stabili-

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* Corresponding authors E-mail: jessylan9963@sina.com

ty that can perform in a full pH range of 1 to 14. Therefore, it is increasingly applied effectively for both research labs and industrial processes [8-10]. However, no report has been published on the use of nanoscale polymer particle for the separation of AMD. The present paper described a very successful method to prepare AMD with high purity from crude extract of *Streptomyces* fermentation broth. The separation conditions were optimized after the investigation.

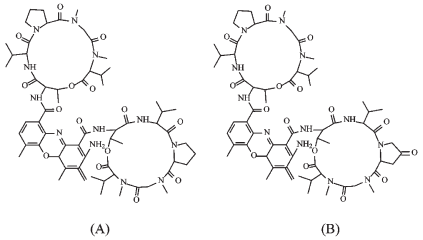


Fig. 1 Chemical structure of actinomycin D (A) and actinomycin S3 (B)

Materials and Methods

Apparatus

MPLC was conducted on an EZ Purify III system (Shang Hai Li Hui Chemical Group Co.); The analytical RP-HPLC was conducted on a Shimadzu 20A DAD series system; Rotary evaporator was from EYELA N-1100, Japan.

Reagents and materials

Nanoscale polymer particles [PS40-300 (based on polystyrene/divinylbenzene media, particle size 40μm, pore size 300Å), PSN40-300 (based on poly divinylbenzene / acrylic media, particle size 40μm, pore size 300Å), PMM40-300 (based on polyacrylic media, particle size 40 μm, pore size 300Å)] were purchased from Jiang Su Nawei Group Co. All organic solvents used for separation were of analytical grade. Methanol used for HPLC analysis were of chromatographic grade (Merck, Whitehouse Station, NJ, USA), and water was distilled before usage. The fermentation broth of AMD was provided by Fujian Institute of Microbiology. The culture broth was subjected to column chromatography over macroporous resin, and eluted with 75% ethanol. The ethanol eluent was concentrated, then the residues were extracted four times with EtOAc to give the crude extract sample for experiment.

Separation procedure of AMD with nanoscale polymer particles

Three nanoscale polymer particles (PS40-300, PSN40-300, PMM40-300) were selected for the separation of AMD. Firstly, these nanoscale polymer particles were soaked in the 0.1 mol/L NaOH for 3 h, and then washed to neutral with water. These pretreated polymer particles were subjected to the column (2.5cm × 40 cm). 0.5 g crude extract sample dissolved in the ethanol were loaded to the column wet-packed with above polymer particles, and the elution conditions, including amount of loading sample, elution speed, and concentrations of eluent were optimized. The effluent was monitored by HPLC at 254 nm and the fractions containing above 95% AMD were collected manually according to the chromatographic peak profiles displayed on the recorder.

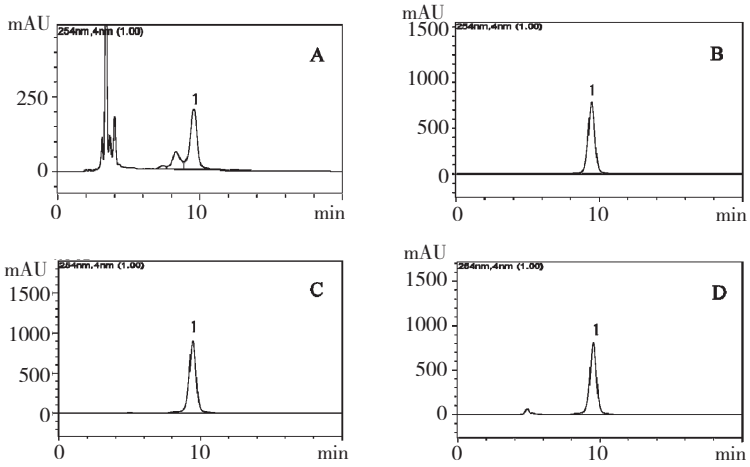
HPLC analysis of the fractions

HPLC analysis was conducted on a Hypersil C_{18} column (250 mm × 4.6 mm i. d., 5 μm) using a methanol-water (72∶28, v/v) as mobile phase, with a flow rate of 1.0 mL/min at 40 ℃. Chromatograms were recorded at 254 nm. The purities of the collected fractions were determined by HPLC based on the peak area of the target species normalized to the sum of all observed peaks.

Results and Discussion

Screening of nanoscale polymer particles

100 mL of the 3 pretreated polymer particles were respectively put into column (2.5 cm × 40 cm) and 0.5 g of crude extract sample (Fig. 2A) dissolved in the 2.0 mL ethanol was added onto the column, and then eluted with 300 mL water and following with 65% ethanol solution at a velocity of 5.0 mL/min. The eluting solution was collected and the purity of AMD was determined by HPLC (Fig. 2B-D). The eluent fractions containing above 95% AMD were pooled, and the volume and content of AMD were recorded as the results shown in the Table 1. From Table 1, it was seen that the elution yield of AMD was the highest at the separation process by PS40-300. AMD was better separated from AMS by using PS40-300.



HPLC conditions; see Section 2.4. Peak 1 correspond to Actinomycin D.

Fig. 2 HPLC chromatograms of Actinomycin D crude sample (A), purified Actinomycin D extracted with PS40-300 (B), PSN40-300 (C) and PMM40-300 (D)

Table 1 The elution yield of AMD by different nanoscale polymer particles

Type of resins	AMD		
	Volume (mL)	Content (μg/mL)	Yield
PS40-300	50.00	4420.35	70.16
PSN40-300	40.00	4529.86	57.52
PMM40-300	40.00	4239.72	53.84

Table 2 Eluting results under different eluent concentrations

Ethanol concentration	AMD		
	Volume (mL)	Content (μg/mL)	Yield
40%	0	–	–
65%	50.00	4420.35	70.16
75%	30.00	4150.21	39.53

Selection of optimal separation process

Ethanol concentration

4 columns (2.5 cm × 40 cm) were selected for studying. 100 mL of the PS 40-300 was put into each column and 0.5 g of crude extract sample dissolved in the 2.0 mL ethanol was added onto the column, and then eluted with 20%, 40%, 65%, and 75% aqueous-ethanol solution at a velocity of 5.0 mL/min, respectively. The eluting solution was collected and the purity of AMD was determined by HPLC. The eluent fractions containing above 95% AMD were pooled, and the volume and content of AMD were recorded as the results shown in the Table 2. It was shown that AMD can not be eluted when the concentration of ethanol was below 40%. If the ethanol concentration was up 75%, AMD and AMS can not be separated well. The eluting effect of AMD was the best with 65% aqueous-ethanol solution.

Amount of loading sample

5 columns (2.5 cm × 40 cm) were selected for studying. 100 mL of the PS40-300 was put into each column and 0.1, 0.3, 0.5, 0.7, 1.0 g of crude extract sample dissolved in ethanol was added onto the column respectively, and then eluted with 65% ethanol solution at a velocity of 5.0 mL/min. The eluting solution was collected and the purity of AMD was determined by HPLC. The eluent fractions containing above 95% AMD were pooled, and the eluting curves are shown in Fig. 3, the data of AMD elution yield expressed in mean ± S.D. ($n = 3$, points, mean; bars, S.D.). It suggested that yield of AMD was lower when the loading sample was below 0.3 g. If the loading sample was up 0.7 g, AMD and AMS can not be separated well. Therefore, the eluting effect of AMD was the best by using the loading sample of 0.5 g/100 mL PS40-300.

Eluting speed

4 columns (2.5cm × 40cm) were selected for studying, 100 mL of the PS40-300 was put into each column and 0.5 g of crude extract sample dissolved in the ethanol was added onto the column, and then eluted with 65% ethanol solution at a velocity of 3.0, 4.0, 5.0, 6.0, 7.0 mL/min, respectively. The eluent

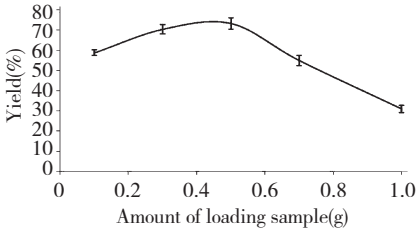


Fig. 3 Elution yield of AMD (mean ± S. D., $n = 3$) for different amounts of sample

fractions containing above 95% AMD were pooled, and the eluting curve was shown in Fig. 4, the data of AMD elution yield expressed in mean ± S. D. ($n = 3$, points, mean; bars, S. D.). It was shown that AMD and AMS cannot be separated well when the eluting speed was up 6.0%. The eluting effect of AMD was the best at a velocity of 5.0 mL/min.

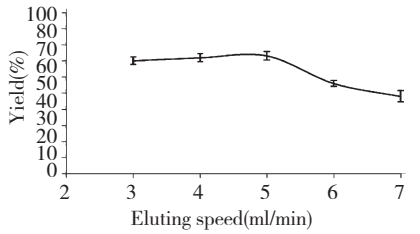


Fig. 4 Elution yield of AMD (mean ± S. D., $n = 3$) at different elution speeds

Amplification of the separation process

Based on the optimal separating conditions, the amplification of the separation process was carried out by MPLC. 400 mL of the pretreated PS40-300 was put into column and 2.0 g of crude extract sample was added onto the column, and then eluted with water, 40%, and 65% ethanol solution respectively at a velocity of 15.0 mL/min. Peak 1 and Peak 2 were collected separately recorded as the results shown in the Fig. 5. The eluent fractions containing above 95% AMD (Peak 2) were pooled.

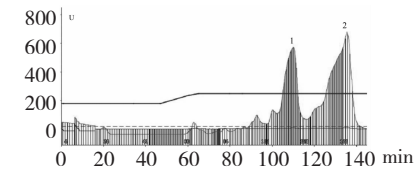


Fig. 5 MPLC chromatogram of the sample solution eluted with PS40-300

Conclusion

In this study, the separation strategy by nanoscale polymer particles PS40-300 was confirmed to be an effective approach for separation of AMD. The factors influencing separation such as the amount of loading sample, elution speed, and different eluent concentrations were optimized. The amplification of the separation process showed that AMD and AMS can be collected separately. The yield of high-purity AMD (above 95%) was up 70%. Therefore, this separation method can be a better choice for the separation of AMD from fermentation broth.

References

1 Waksman SA, Woodruf HB. Actinomyces antibiotics, a new soil organism antagonistic to pathogenic and nonpathogenic bacteria. *J Bacteriol*, 1941, 42: 231-249.

2 Liao AF(廖爱芳), Liu Y(刘勇), Wang JM(王建民), *et al*. Screening of actinomycin D producing strain *S. Luteolutescens* N45. *Chin Pharm Biotech*(药物生物技术), 2002, 9(2): 88-90.

3 Yu L(郁蕾), Dai HF(戴好富), Wei J(魏景), *et al*. Bioassay-guided isolation and identification of antibacterial component of *Stretomyces costaricanus*. *Chin J Antibiotics*(中国抗生素杂志), 2011, 36: 507-510.

4 Farber S, D'Angio G, Evans A, *et al*. Clinical studies on actinomycin D with special reference to Wilms' tumor in children. *J Urol*, 1960, 2002: 2560-2562.

5 Huang CC, Hou Y, Wang JJ. Effects of a new antitumor agent, epipodophyllotoxin, on growth and chromosomes in human hematopoietic cell lines. *Cancer Res*, 1973, 33: 3123-3129.

6 Osathanondh R, Goldstein DP, Pastorfide GB. Actinomycin D as the primary agents for gestational trophoblastic disease. *Cancer*, 1975, 36: 863-866.

7 Lin WL(林文良), Fang JZ(方接枝), Huang WZ(黄维真). Study on the chemical structure of actinomycin 23-21. *Chin J Antibiotics*(中国抗生素杂志), 1980, 5: 1-5

8 Lin Y(林毅), Zhang XX(张雪霞), Li N(李宁), *et al*. Studies on the separation and purification of daptomycin by nanoscale polymer particles. *Chin J Antibiotics*(中国抗生素杂志), 2013, 38: 588-591.

9 Li XL(李晓露), Ren FZ(任风芝), Wang HY(王海燕), *et al*. Separation and purification of pneumocandin A0 and B0 with nanoscale polymer particles. *Chin J New Drug*(中国新药杂志), 2014, 23: 344-346.

10 Ren FZ(任风芝), Zhang XX(张雪霞), Chen SH(陈书红), *et al*. Purification and identification of (γ-hydroxylmethylleucine 4)-cyclosporin A. *Chin J Antibiotics*(中国抗生素杂志), 2013, 38: 512-514.