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RESEARCH PAPER

Process Development for Purifying Tacrolimus from *Streptomyces* sp. Using Adsorption

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Abstract The immunosuppressant tacrolimus produced by *Streptomyces* sp. TST10 was purified in a process that included extraction, pre-adsorption using HP20 resin, adsorption using CG161M resin, and crystallization. In this study, the purification process using the adsorption resin CG161M was optimized by correlating tacrolimus yield with analogue load. One-step adsorption and two-step adsorption using CG161M can be applied selectively to the purification process, according to the analogue load of the input solution. We determined a correlation between the analogue load and the first adsorption yield in the two-step adsorption. We also observed yields according to the analogue loads in the one-step adsorption and the second adsorption of the two-step adsorption. As a result, the purification yields can be predicted by input conditions (analogue load). The purification strategy can be modified to achieve specific goals of purity, yield, and cost.

Keywords: tacrolimus, purification, adsorption, analogue, CG161M resin

1. Introduction

An immunosuppressant, tacrolimus (molecular formula: $C_{44}H_{69}NO_{12}\cdot H_2O$) was discovered by the Fujisawa Pharmaceutical Co., which merged with Astellas Pharma in 2004, and is also known as FK-506 or Fujimycin [1,2]. The commercial products derived from tacrolimus are Prograf for the prevention of transplant rejection and Protopic, which is a topical ointment. The annual sales of Prograf were over US$2 billion in 2009, and Prograf ranked as the world's 54th best-selling prescription medicine in the world [3].

Tacrolimus is produced through the fermentation of *Streptomyces*, and in early studies its final titer in broth reached 50 mg/L [1]. Many scientists attempted to increase tacrolimus production by screening new microorganisms, optimizing fermentation, and developing new strains, eventually reaching a final titer 972 mg/L [4-6]. Meanwhile, there have been relatively few studies examining tacrolimus purification, and since these studies may have been reported in the form of patent applications, information about tacrolimus purification is limited. In early studies, tacrolimus purification was a complicated process requiring cell separation, acetone extraction, cell debris removal four rounds of adsorption, ethyl acetate extraction and crystallization, producing a tacrolimus yield of 20% or less [1].

When tacrolimus is produced through the fermentation of *Streptomyces*, analogues that are similar in effect and structure to tacrolimus, as shown in Fig. 1, are also produced [7,8]. Ascomycin (also known as FK-520) is a typical analogue of tacrolimus [9]. Because analogues with structures similar to that of tacrolimus are difficult to remove with normal adsorption resin, purification using an adsorption resin bound with silver ion [10,11] and purification by HPLC (high-performance liquid chromatography) [12] have been developed to achieve effective removal of the analogues. In the former method, the cost of silver ion is high, and a subsequent purification step is needed to remove it. The latter method is not appropriate for commer-

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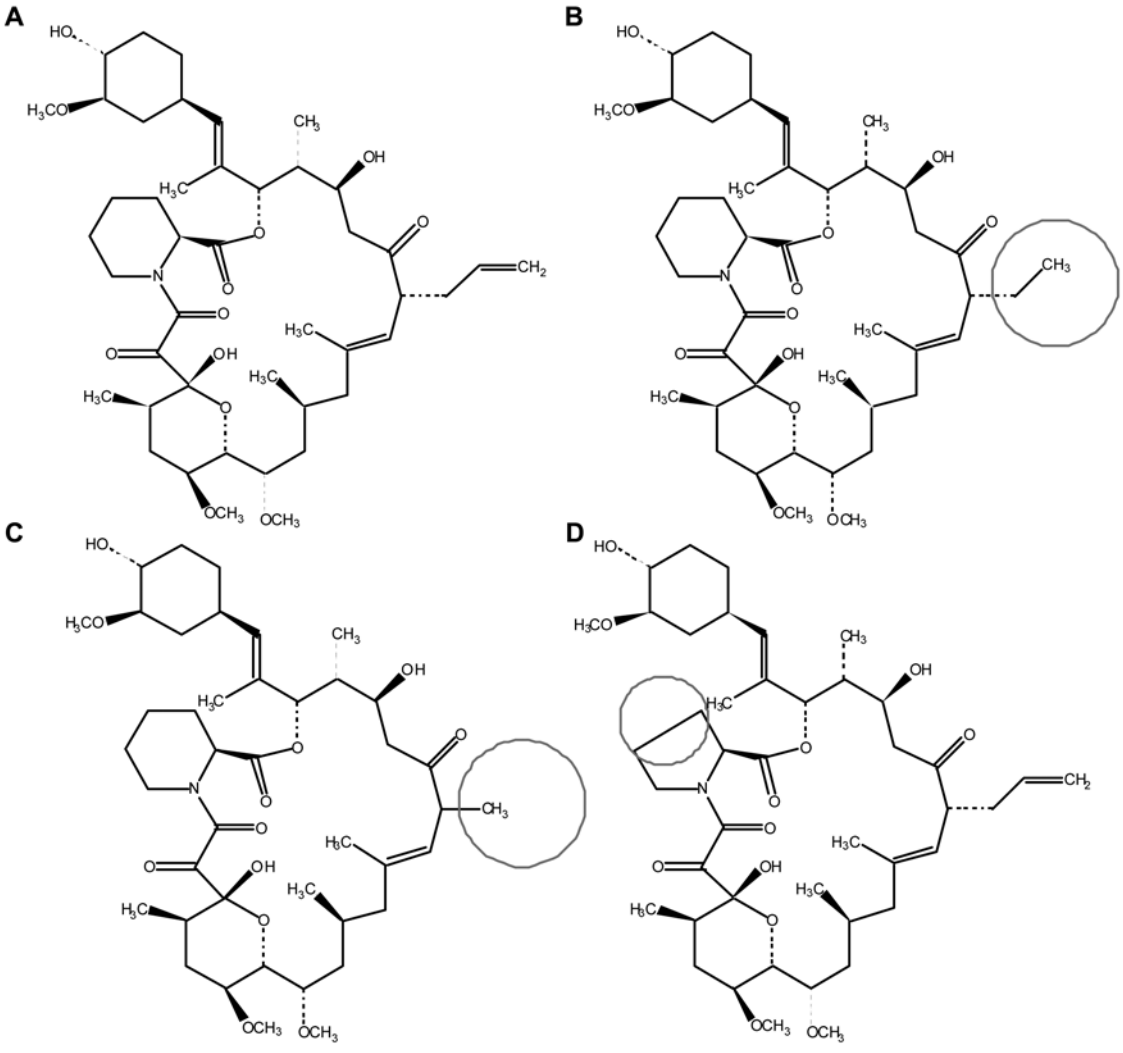

Fig. 1. Tacrolimus (FK-506) and its analogues. (A) FK-506, (B) FK-520, (C) FK-523, and (D) FK-525.

cial production because of the high price and low throughput of HPLC. However, an economical purification process with crystallization, adsorption, and second crystallization was developed to produce high-purity tacrolimus [13]. This process would be compatible with large-scale commercial production because it involves a comparatively simple process and results in a moderate yield of 30 ~ 35%, but it may not cope with various analogue concentrations of input solution in the process using crystallization as the main purification step.

CG161M is a macroporous chromatographic resin consisting of an insoluble polystyrene divinylbezene polymer with a mean diameter of 75 μm and is widely used for laboratory- and process-scale purification of proteins, peptides, nucleic acids, antibiotics, and small molecular weight pharmaceuticals [14].

We developed a process for purifying tacrolimus from *Streptomyces* sp. to obtain high-purity tacrolimus at high yield, removing analogues effectively, and minimizing tacrolimus loss according to various input solutions.

2. Materials and Methods

2.1. Fermentation

We used *Streptomyces* sp. TST10, which was isolated and developed at the TS Corporation in Korea for tacrolimus fermentation. TST10 was cultured at 27 ºC for seven days in a 5-liter jar fermenter (KoBioTech, Korea) to produce tacrolimus after two-step seed culture. The production culture medium consisted of oxidized starch, soybean meal, inactive dry yeast, $(NH_4)_2SO_4$, and the surfactant GE304 [6].

2.2. Tacrolimus purification

The final broth of the production culture was mixed with

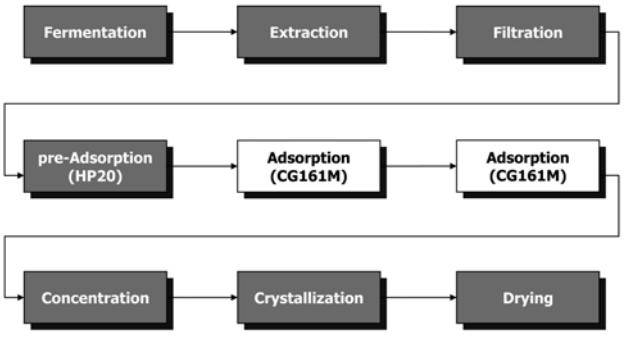

Fig. 2. The purification process used for tacrolimus production.

an equal volume of acetone and extracted, and solid parts were removed by filtering. Colored material and impurities, except analogues, were removed by pre-adsorption using HP20 resin (Samyang, Korea), and analogues were removed by adsorption using CG161M resin (Dow). The pre-adsorption using HP20 was composed of equilibration with distilled water, loading, washing with 55% acetone solution, and elution with 75% acetone solution. Solution after the adsorption using CG161M was concentrated by vacuum evaporation at 45 °C and chilled at 4 °C for crystal growth. The tacrolimus crystal was filtered and dried. The filtrate was recycled for re-crystallization to reach a yield over 95%. Crystalline tacrolimus with purity of greater than 98% was obtained through the crystallization of the purified solution (Fig. 2).

2.3. Purification using CG161M

In previous studies, we could not find conditions for CG161M purification in which analogues were fully separated from tacrolimus, but we determined a condition in which the peaks of tacrolimus and analogue concentrations in the elute fraction of CG161M separated. Therefore, a purification process using CG161M was conducted at this condition. The solution after the pre-adsorption using HP20 was adjusted to an acetone concentration of 42%, and the solution was loaded into a column packed with 1,000 mL CG161M equilibrated with a 40% acetone solution. After loading of the solution, unbound materials were washed with three resin volumes of 40% acetone solution and bound materials were eluted with 25 resin volumes of 52.5% acetone solution. The flow rate of the solutions was 1 resin volume per hour. Eluted fractions were collected with one resin volume and analyzed by HPLC. The fractions were selected based on their tacrolimus purities and mixed to achieve the desired purity of tacrolimus. The CG161M resin, after purification, was washed with 100% acetone and equilibrated with 40% acetone for the next process.

2.4. Analysis

Each fraction was analyzed by HPLC (Agilent) on a Synergi Polar RP column (4 μm, 250 × 4.6 mm, Phenomenex, Macclesfield, UK) at 55 °C with 1 mL/min 50% acetonitrile effluent and a 220 nm UV detector. Tacrolimus and ascomycin from LC Laboratories (Boston, USA) were used as standard materials and an analogue with a peak that appears after tacrolimus at a retention time of about 26 min at the above conditions was determined to be the analogue RT26.

3. Results and Discussion

3.1. Characteristics of purification using CG161M

Tacrolimus with high purity could not be obtained by the adsorption method using HP20 because non-analogue impurities were removed efficiently, but the ratio of analogues to tacrolimus was maintained in the HP20 purification. The tacrolimus yield was not affected by the HP20 purification, which did not result in the loss of tacrolimus.

When tacrolimus was purified using CG161M after pre-adsorption using HP20, ascomycin was eluted before tacrolimus and RT26 was eluted after tacrolimus, but they were not fully separated, as depicted in Fig. 3. The fractions with relatively high analogue concentrations should be removed to obtain high-purity tacrolimus. At the same time, however, tacrolimus in the removed fractions was also removed and the tacrolimus yield decreased. The sum of the ratios of ascomycin and RT26 to tacrolimus (the analogue ratio) should be less than 1.2% after CG161M purification, considering that the final purity of tacrolimus should be more than 98% and that there might be other analogues, some of which may be removed by the subsequent crystallization step. To reach the target analogue

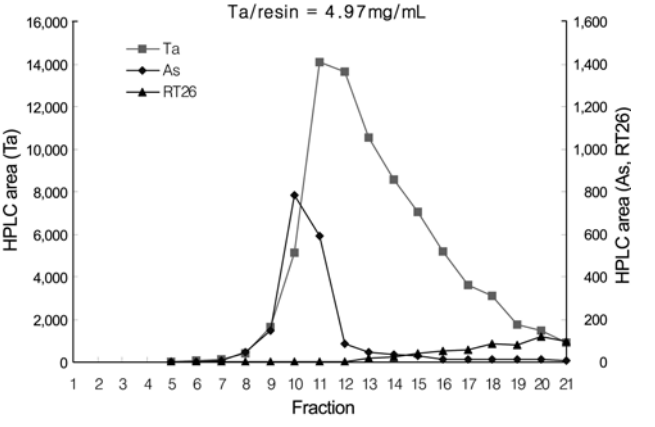

Fig. 3. Elution characteristics of tacrolimus, ascomycin, and RT26 during the CG161M purification process.

Table 1. Summary of loading solutions used in this study

Test no.	Tacrolimus/ Resin (mg/mL)	Ascomycin/ Tacrolimus (1)	RT26/ Tacrolimus (2)	Analogue ratio (1) + (2)
1	2.28	2.2%	1.1%	3.3%
2	2.74	2.5%	1.5%	4.0%
3	3.01	7.2%	3.9%	11.2%
4	3.38	2.5%	1.3%	3.8%
5	3.84	7.2%	2.6%	9.8%
6	4.28	2.5%	1.2%	3.7%
7	4.63	7.1%	4.9%	12.0%
8	4.87	8.2%	7.6%	15.9%
9	4.97	2.1%	1.4%	3.5%
10	5.44	3.0%	1.1%	4.1%
11	6.28	6.8%	3.1%	10.0%
12	6.90	5.5%	2.6%	8.0%
13	8.11	5.7%	3.5%	9.2%
14	8.76	7.0%	3.2%	10.2%
15	8.84	13.0%	7.2%	20.1%
16	9.03	11.3%	6.0%	17.3%

ratio after CG161M purification, one-step or two-step adsorption using CG161M was applied selectively, according to the quantity of tacrolimus and the analogue ratio of the CG161M loading solution.

3.2. One-step adsorption using CG161M

Sixteen solutions with various quantities of tacrolimus and analogue ratios were purified using CG161M. The ratios of tacrolimus to resin volume (tacrolimus loads) and analogue ratios are summarized in Table 1.

When tacrolimus yields were calculated by selecting and mixing fractions to reach the target analogue ratio of 1.2%, eight solutions were purified, whereas the others were not purified (yield 0%). The tacrolimus yields indicated a weakly negative correlation with the tacrolimus loads, ascomycin to tacrolimus, RT26 to tacrolimus, and the analogue ratios, but the tacrolimus yield could not be predicted by the correlation. Fig. 4 depicts the tacrolimus yields plotted according to analogue loads (products of tacrolimus load and analogue ratio, (As+RT26)/resin). The tacrolimus yields have some correlation with the analogue loads and are divided in three groups, according to the analogue loads. Group 1 (G1), with analogue loads of 0 ~ 0 .2 mg/mL, demonstrates yields of over 60%. Group 2 (G2), with analogue loads of 0.2 ~ 0.4 mg/mL, exhibits yields of 20 ~ 40%. Group 3 (G3), with analogue loads of over 0.5 mg/mL, indicates a yield of 0% and could not be purified through one-step adsorption using CG161M. The fraction of one column volume may cause the stepwise differences of the yields among groups, which can be reduced by reducing the fraction volume. G1 is a loading condition

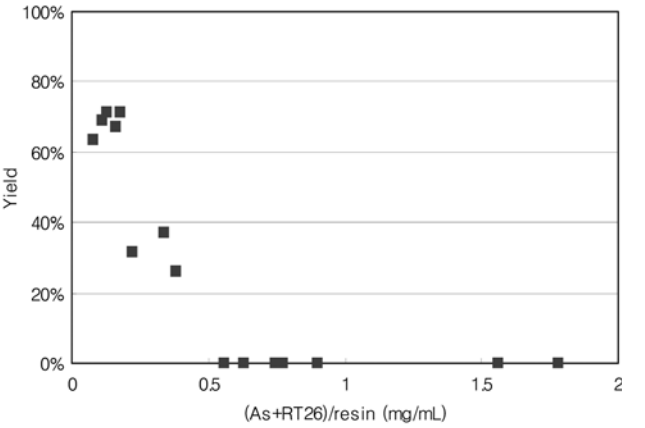

Fig. 4. Results of one-step adsorption using CG161M.

with high yield by one-step adsorption, and G2 is a loading condition in which one-step or two-step adsorption using CG161M can be selectively applied, depending on the economics of the process. Meanwhile, G3 should be purified in the two-step adsorption or reduced in loading volume to decrease the analogue load for the one-step adsorption. An analogue ratio of 3.7%, the average analogue ratio of G1, is suitable for a target analogue ratio of the first adsorption in the two-step adsorption, and the predicted yield of the second adsorption in the two-step adsorption is 68.5%. Table 2 summarizes tacrolimus yields according to analogue loads of input solution and target analogue ratios (1.2%, 3.7%).

3.3. Two-step adsorption using CG161M

Table 2. Tacrolimus yields of CG161M purification according to analogue loads of input solution and target analogue ratios

Test no.	Tacrolimus/Resin (mg/mL)	Yield	
		Target analogue ratio 1.2%	Target analogue ratio 3.7%
1	0.08	63.6%	
2	0.11	69.0%	
4	0.13	71.4%	
6	0.16	67.1%	
9	0.17	71.5%	
10	0.22	31.8%	87.1%
3	0.34	37.4%	78.9%
5	0.38	26.4%	86.1%
12	0.55	0.0%	69.1%
7	0.56	0.0%	52.8%
11	0.63	0.0%	61.1%
13	0.74	0.0%	68.2%
8	0.77	0.0%	44.1%
14	0.90	0.0%	28.7%
16	1.56	0.0%	0.0%
15	1.78	0.0%	0.0%

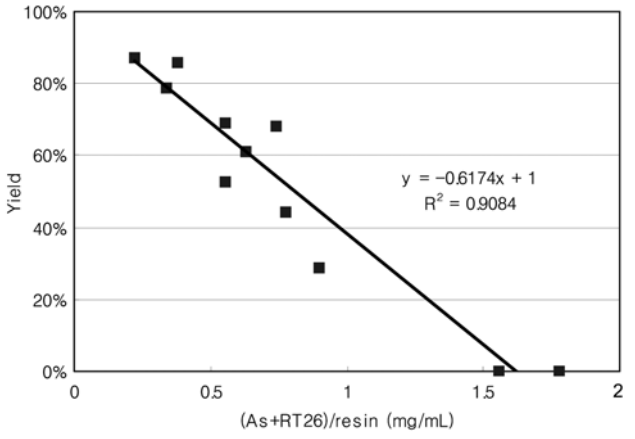

Fig. 5. Results of the first adsorption in the two-step adsorption step using CG161M.

Because the tacrolimus concentration and analogue ratio of the final broth vary according to the *Streptomyces* sp. TST10 fermentation, an appropriate CG161M purification strategy is required for the various broths. As a result of the one-step adsorption using CG161M, it was found that solutions with analogue loads of less than 0.2 mg/mL could be purified with high yield in the one-step adsorption, and solutions with analogue loads of more than 0.2 mg/mL required a two-step adsorption using CG161M for high yield. Fig. 5 exhibits yields of G2 and G3 in the first adsorption of the two-step adsorption plotted according to analogue loads, where the target analogue ratio was 3.7%. The yields and analogue loads have a negative correlation, and the equation is as follows;

$$Y = 1 - k(As+RT26)(Ta) \quad (1)$$

where Y: tacrolimus yield
k: constant, 0.6174/(mg/mL)
As: ratio of ascomycin to tacrolimus in the loading solution
RT26: ratio of RT26 to tacrolimus in the loading solution
Ta: tacrolimus load, mg/mL.

Yields from the first adsorption in the two-step adsorption can be calculated using analogue loads and equation (1). To reach a commercially feasible yield of 40%, the yield of the first adsorption in the two-step adsorption should be over 58.4%, given the yield of the second adsorption in the two-step adsorption (68.5%) and that the analogue load should be under 0.67 mg/mL. When the analogue load of the input solution is over 0.67 mg/mL, the solution can be purified by reducing the quantity of input solution or increasing the CG161M resin.

The analogue ratio after the first adsorption in the two-step adsorption is fixed at 3.7%, but the analogue load varies according to the yield of the first adsorption in the two-step adsorption. If the same CG161M column is used for the first and second adsorption steps in the two-step adsorption, the number of second-adsorption steps will be effectively reduced by adjusting the loading solution volume to achieve the proper analogue load of 0.2 mg/mL. Concerning test #5, which could be purified in one-step or two-step adsorption using CG161M, the yield of the one-step adsorption was 26.4%, and that of the two-step adsorption was 59.0% (with a first yield of 86.4% and second yield of 68.5%). The overall yield of test #5, where two-step adsorption using CG161M was applied was 56.1% considering the pre-adsorption yield of 100% and the crystallization yield of 95%. The yield of the two-step adsorption was two times more than that of the one-step adsorption. It would be possible to employ the one-step adsorption for relatively cheap and simple processes or the two-step adsorption for high-yield applications, as determined by cost-benefit analysis.

4. Conclusion

We developed a process for purifying tacrolimus from *Streptomyces* sp. using the adsorption resin CG161M. The tacrolimus yield from the adsorption using CG161M was affected by the tacrolimus load and the analogue ratio of the input solution, and correlated well with the analogue load. Input solutions with analogue loads of less than 0.4 mg/mL were purified by the one-step adsorption to reach the target analogue ratio of 1.2%, and correlations between yields and analogue loads were found. The two-step adsorption was applied for input solutions that could not be purified by the one-step adsorption, and we found a correlation equation between the yields and the analogue loads of the first adsorption in the two-step adsorption. The yield of the second adsorption in the two-step adsorption could be predicted by the correlation of the yields and the analogue loads employed in the one-step adsorption. As a result, we can predict the tacrolimus yield of CG161M purification from the equation and correlations, and modify the tacrolimus-purification strategy for various input solutions to achieve specific goals of purity, yield, and cost.

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