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Stabilization Effect of Resin on the Production of Potent Proteasome Inhibitor NPI-0052 During Submerged Fermentation of *Salinispora tropica*

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Abstract Addition of acrylic resin Amberlite XAD-7 during the fermentation of *Salinispora tropica* significantly enhanced the production of NPI-0052 by 69 fold. Examination of the time course of resin addition to the *Salinispora tropica* fermentation demonstrated that the increase in the production of NPI-052 is due to the stabilization effect by resin but not the removal of an end product feedback repression. Delay in resin addition to the fermentation led to decreases in the production of NPI-0052 to the amounts that are synthesized prior to the resin addition.

Keywords NPI-0052, proteasome inhibitor, *Salinispora tropica*, Amberlite XAD-7 resin

NPI-0052 (salinosporamide A) is a novel, potent proteasome inhibitor [1～3] isolated from the marine obligate actinomycete *Salinispora tropica* [4]. It possesses a broad spectrum of activities against various tumors in animal models [1, 2, 5]. It is currently undergoing Phase I clinical studies for the treatment of patients with various cancers [2]. Structural studies revealed that NPI-0052 comprises a fused γ-lactam-β-lactone ring system that is decorated with a cyclohexenyl carbinol at the C-4 ring junction, a chloroethyl substituent at C-2, and a methyl group at the C-3 ring junction (Fig. 1) [4]. Structure-activity relationship and mechanistic studies indicated that the β-lactone ring system and the chlorine atom are required for the potent activity of NPI-0052 [3, 6]. The β-lactone ring system is susceptible to hydrolysis in an aqueous environment [6, 7], such as the submerged fermentation condition for the production of NPI-0052 by *S. tropica*. End product feedback repression of secondary metabolite biosynthesis is a well known phenomenon [8]. The extremely potent activity of NPI-0052 may lead to end product repression of its biosynthesis, which would limit the biosynthesis of NPI-0052 by *S. tropica*. These factors may be responsible for the initial low production of NPI-0052, about 4.0 mg/liter, in the submerged fermentation of *S. tropica*.

It has been well documented that the addition of resins to the fermentations of reactive and/or highly potent secondary metabolites led to increases in production of these metabolites [9～12]. Therefore, we examined the effect of Amberlite XAD-7 resin on the production of NPI-0052 by *S. tropica*. This communication describes the

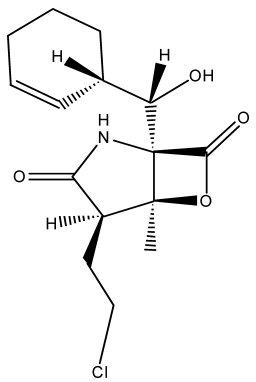

Fig. 1 Structure of NPI-0052.

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increase in the titer of NPI-0052 produced by *S. tropica* as a result of resin addition.

Materials and Methods

Microorganism

The producing strain, *S. tropica* NPS21184 was deposited with the American Type Culture Collection and assigned the accession number PTA-6685.

Growth Media and Culturing Conditions

The growth media and culturing conditions have been described previously [13] with the modification of resin addition schedule.

Growth Analysis, Extraction and Analytical Methods

The growth analysis by packed cell volume, the preparation of fermentation extract for HPLC analysis and the HPLC conditions for monitoring the production of NPI-0052 have been described previously [13]. The growth of culture was expressed as % packed cell volume defined as volume of packed cell/volume of culture×100%.

Results and Discussion

Production of NPI-0052 at Different Times of Resin Addition

Denora *et al.* [7] demonstrated that NPI-0052 is rapidly degraded in the aqueous environment such as phosphate buffer (0.05 M) at pH 6.5 and 25°C with a $t_{50\%}$ ~140 minutes. This may explain the low titer of NPI-0052 observed in the submerged fermentation of *S. tropica*. Preliminary study was carried out to determine the effect of resins in enhancing the production of NPI-0052 by adding Amberlite XAD-4, Amberlite XAD-7 and Amberlite XAD-16 resin to the shake flask cultures at 24 hours at a final concentration of 20 g/liter. It was determined that Amberlite XAD-7 was the most effective resin tested in increasing the production of NPI-0052 (Table 1). It was also determined that increasing the concentration of Amberlite XAD-7 in the fermentation to 30 g/liter did not improve the production of NPI-0052, indicating that the final concentration of resin at 20 g/liter was optimal for the current production of NPI-0052 in shake flask culture. Amberlite XAD-7 resin was selected for the time course study. Table 2 shows the production of NPI-0052 in shake flask cultures with the acrylic Amberlite XAD-7 resin (20 g/liter final concentration) added at 24, 48, 72 or 96 hours, and the control culture with no resin addition. In the control culture, the production of NPI-0052 was first observed at 48 hours. The peak production of 4.0 mg/liter was observed at 72 hours in the control culture and continued to drop to 2.0 mg/liter and 0.1 mg/liter at 96 hours and 120 hours, respectively. When resin was added to the culture at 24 hours, we observed a significant increase in the production of NPI-0052, with the peak titer of 275 mg/liter detected at 96 hours. This corresponds to a 69-fold increase in the production of NPI-0052 as compared to the control culture. This observation is similar to the yield improvement by resin on production of the highly reactive and the most potent antitumor agents esperamicin and

Table 1 Effect of Amberlite XAD-4, Amberlite XAD-7 and Amberlite XAD-16 resin on the production of NPI-0052 in shake flask cultures of *Salinispora tropica*

Resin	NPI-0052 (mg/liter)
No addition	5.7
Amberlite XAD-4 (20 g/liter)	207
Amberlite XAD-7 (20 g/liter)	278
Amberlite XAD-16 (20 g/liter)	218

Table 2 Time course of NPI-0052 production in shake flask cultures of *Salinispora tropica* with different times of Amberlite XAD-7 resin addition

Time of resin addition	NPI-0052 (mg/liter)					
	24 hours	48 hours	72 hours	96 hours	120 hours	144 hours
No addition	0	3	4	2	0.1	0.1
24 hours	0	62	233	275	245	222
48 hours	ND	3	158	224	219	178
72 hours	ND	ND	4	108	99	92
96 hours	ND	ND	ND	2	15	10

ND=Not determined.

Table 3 Correlation of titer of NPI-0052 in shake flask cultures of *Salinispora tropica* with different times of Amberlite XAD-7 resin addition

Time of resin addition	Maximum titer (mg/liter)	Difference in titer as compared to 24 hours resin addition (mg/liter)	Amount of NPI-0052 synthesized since resin addition at 24 hours (mg/liter)
24 hours	275	—	—
48 hours	224	51	62
72 hours	108	167	158

dynemicin of the enediyne class [9, 10]. In that system, the resin showed both stabilization and removal of feedback repression of esperamicin and dynemicin biosynthesis. Based on the improvement in yield shown for Amberlite XAD-7 with NPI-0052, and since this molecule is a highly reactive and extremely potent antitumor agent, one might hypothesize that this resin showed both stabilization and removal of feedback repression effects on the production of NPI-0052 also.

Mechanism of the Effect of Resin on the Production of NPI-0052

In order to elucidate the mechanism of the effect of resin on the production of NPI-0052, we compared the production of NPI-0052 with different times of resin addition to the culture. When resin was added to the cultures later than 24 hours, the peak production of NPI-0052 was decreased. The later the time of resin addition, the lower the peak titers of NPI-0052. The peak titer of 275, 224, 108 and 15 mg/liter were detected at 24, 48, 72 and 96 hours resin addition, respectively (Table 2). The drop in the production of NPI-0052 when the resin was added later than 24 hours can be accounted for by the loss of NPI-0052 synthesized during the period by degradation (hydrolysis) in the absence of resin (Table 3). From 24 to 48 hours, the culture was capable of producing 62 mg/liter of NPI-0052, which can be accounted for by the drop in titer of 51 mg/liter when resin was added to the culture at 48 hours instead of 24 hours (Table 3). By the same token, the drop in titer of 167 mg/liter at 72 hours resin addition as compared to 24 hours resin addition was due to the loss of the synthesis of 158 mg/liter by degradation during the 48 hours period (*i.e.*, from 24 to 72 hours) (Table 3). The above observation clearly demonstrated that the increase in the production of NPI-0052 is due to the stabilization effect by resin but not by removal of end product feedback repression. The presence of 3 to 4 mg/liter unbound NPI-0052 in the culture did not exert any adverse effect on further production of NPI-0052.

Effect of Resin Addition on the Growth Yield and pH of the Fermentation

Different time of resin addition to the shake flask culture did not affect the growth of the organism and the pH of the fermentation. The growth yield and the pH were essentially the same throughout the fermentation in the control culture and the cultures with different times of resin addition. At 96 hours (peak NPI-0052 production), all cultures have the same packed cell volume (6.0%) and the same pH range of 7.3 to 7.4.

Conclusion

We established that the optimal time of resin addition to the shake flask culture for NPI-0052 production is 24 hours of the production cycle. Resin acted as the "stabilization agent" by binding to NPI-0052 to minimize the hydrolytic effect of the aqueous environment during the submerged fermentation of *S. tropica* and increased the production of NPI-0052 by 69-fold.

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