생명과학회지
Korean J. Life Science, 7(3), 186-191, 1997

Isolation of S-627(A) and (B) as Candidates of Anticandiosis Agent

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Abstract

In order to select new anticandiosis agent-producing candidates, we used a modified selection method. Two active fractions designated as S-627(A) and (B) were isolated from the fermentation broth of *Streptomycetes* sp. S-627 which was screened out using the characteristics of the dimorphic *Candida albicans* K-1. S-627(A) and (B) were partially characterized by methanol extraction, diaion HP-20 and silica gel column chromatography (chloroform-methanol, and benzene-ethyl acetate), and preparative thin layer chromatography.

Key words : *anticandiosis agent, purification, fermentation broths*

Introduction

The development of new classes of antifungal agents is an important mission for the future. Many different species of *Candida* can cause disease, but *C. albicans* is responsible for of serious fungal infection in most cases[1].

Diseases caused by *C. albicans* are termed candiosis[2]. The diseaseUs symptoms range from chronic infections affecting the skin to acute infections of deep tissues. The microbe has been isolated from more than 80% of specimens obtained from women with vulvovaginal candiosis[3]. The cellular structure and function of *C. albicans* are similar to those of mammalian cells. These similarities present major problems when developing antifungal compounds which are active for candiosis[4]. Individuals who are immunosuppressed may acquire a fungal infection following exposure to weakly pathogenic organisms that occur as saprophytes in the environment. Infections such as candiosis, aspergillosis, histoplasmosis, and coccidiomycosis are increasingly being seen in immunocompromised patients, particularly ones with the acquired immune deficiency syndrome (AIDS) or those with haematological malignancies[5-11]. In comparison with the number of antibacterial drugs available, there are far fewer antifungal compounds. Even so, the number of antifungals are increasing all the time. There are three major families of drug types : the azoles, the polyenes, and the allylamine[12]. In addition to this, there is a miscellaneous group of compounds such as flucytosine and griseofulvin. A few types of drugs which interfere with cell wall synthesis in *C. albicans* are under development constantly, but have not yet been developed in a sufficient spectrum for the inhibition of the cell wall[12-13].

In this study, in order to develop more powerful candidates of the anticandiosis agent, we used the dimorphic *C. albicans* K-1 as a test organism. Consequently, two active fractions, S-627(A) and (B) were screened

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out, and partially characterized.

Materials and Methods

General experimental procedures

Kiselgel 60 (Merck) and Sephadex LH-20 (Pharmacia) were used for column chromatography and DC-Fertigplatten Kieselgel 60 (Merck) was used in TLC analysis.

Culture and medium

C. albicans K-1, from a clinical isolate (a donation of Kyungpook National University Hospital, Taegu, Korea ; data not shown) was maintained and stocked on sabouraud dextrose agar slants at 4℃. A large loopful of microbes was inoculated in yeast nitrogen broth (Difco, MI) and incubated for 1 day at 37℃. The sabouraud dextrose agar plate was inoculated with the cell suspension and incubated overnight at 37℃. Cells were counted in a haematocytometer and adjusted to a concentration of 1×10^7 cells/ml.

Prescreening

C. albicans K-1 was precultured in sabouraud broth at 30℃ for 24 h. The cells were then harvested with a effendorf centrifuge at 12,000 rpm for 5 min and then washed with the broth. After adjusting the cells to the concentration of 1.0×10^8 cells/ml, the plate was prepared and hardened to a final concentration of 1% seed in Eagles Minimum Essential Medium supplemented with 5% heat-inactivated fetal calf serum (Life Technology, NY). The inhibition zone of the antimicrobial spectrum of various fermentation broths was determined using a 6 mm paper disc (Abott, IL) on the MEM agar plate in a humidified atmosphere of 5% CO_2 in air at 37℃. The sabouraud agar plate was also prepared and loaded as a control for the comparison of the inhibition zone at normal conditions.

Isolation of microbes

After treatment of the soil sample, actinomycetes were isolated on the agar plate of MYP medium which consisted of 0.5% starch, 0.5% glucose, 0.1% NaCl, 0.1% yeast extract, 0.5% polypeptone, 0.05% K2HPO4, 0.02% $MgSO_4\cdot7H_2O$ per 1 L of deionized water (pH 7.2 before sterilization). The selected isolates were stocked in a −86℃ deep freezer (Forma Scientific) in a Nunc vial with 20% of glycerol.

Fermentation studies

The antibiotic production by *Streptomyces* S-627 was initiated by following procedure. The inoculum was prepared by culturing it in 250 ml-erlenmeyer flasks containing 50 ml of medium. The seed medium consisted of 2% soluble starch, 1% soybean flour, 0.3% NaCl, and 0.3% $CaCO_3$. This medium was prepared with deionized water and adjusted to pH 7.0 before sterilization. The seeded flasks were incubated for 72 h at 28℃ on a rotary shaker at 200 rpm. The fermentation medium consisted of 1.0% starch, 0.2% glucose, 0.5% pharmamedia, 0.1% ammonium sulfate, 0.1% potassium phosphate dibasic, 0.05% magnesium sulfate, 0.1% calcium chloride 0.3% sodium chloride, 10 mg ferric choride per liter and palm oil as an antifoaming agent. The antifoaming agent was added initially at 0.01% and was thus available on demand. This medium was also prepared with distilled water and adjusted to pH 7.0 before sterilization Fermentation was carried out in a 10-liter vessel (New Brunswick Scientific) containing 7 liters of medium.

Taxonomic studies

To investigate the physiological characteristics, the International Streptomycetes project (ISP) media recommended by Shiriling and Gottlieb[14] and those recommended by Waksman[15] were used.

Antibiotics

Cefotaxime sodium salt (Wako, Tokyo, Japan), 5-fluorocytosine (Sigma), ketoconazole, nystatin (Sigma), and ampotericin B (Sigma) were purchased from the suppliers. The other reagents were commercially available.

Results and Discussion

Prescreening

To select the adequate standard agents required to score the inhibition zone of various fermentation cultures, we used a modified prescreening method. As shown in Table 1, ampotericin B was a good compound against *C. albicans* K-1 in MEM agar under 5% CO_2 incubation. Nystatin was also a candidate, but was not competent in the inhibition zone compared to ampotericin B. Table 2 shows an interesting result : namely, activity only in the MEM agar. About 1,000 culture broths had their own activity in both the MEM agar and the SAB agar, but S-626, S-627, and S-135 produced some antibiotics into the broth only using MEM medium under the condition of the atmosphere of 5% CO_2 in air at 37℃. This is an unique result in comparison to other research

Table 2. Biological profiles of various fermentation cultures against the dimorphic *C. albicans* K-1.

Strain No.	SAB agar	MEM agar
134	+*	++(17.0)**
135	−	+(12.0)
136	+	++(15.0)
141	++	+++(18.0)
144	++	+(12.0)
157	+	++(16.0)
612	+	+(15.0)
626	−	+(13.0)
627	−	++(14.0)
631	++	++(17.0)
795	−	+(10.0)
800	+++	+++(18.0)
802	−	+(10.0)
803	++	+(2.0)
932	++	++(14.0)

*−, no activity ; the diameter of 10-13 mm (+), 14−17 (++), 18−21(+++), respectively.

**the number of parenthesis means the diameter of inhibition zone in MEM agar. See Materials and Methods or Table 1 for details.

Table 1. Comparison of activity between various antibiotics against *C. albicans* K-1.

Antibiotic	Conc.*	Inhibition zone(mm)		Inhibition mode	
		MEM agar	SAB agar	at MEM	at SAB
Cefotaxime	100	−	(21.0)	NMC[1]	NT[2]
5-fluorocytosine	100	(20.0)	−	Swelling	Swelling
Ketokonazole	100	−	(32.0)[3]	NMC	NT
Nystain	100	16.0	10.0	Swelling	Swelling
Polyoxin B	100	−	−	NMC	NT
Itulin A	100	−	−	NMC	NT
Amphotericin B	100	19.0	15.0	Swelling	Swelling

* : μg/mℓ

[1]NMC ; no morphological change, [2]NT ; not tested, [3]() ; unclear inhibition zone.

After adjusting the precultured cells to the concentration of 1.0×10^8 cells/ml, plates were made at the final concentration of 1% seed in Eagles Minimum Essential Medium supplemented with 5% FCS. Various culture broths were applied on 6 mm paper disc on the MEM agar plates and the sabouraud agar plates, respectively. Inhibition zone was checked at every 24 h-intervals and measured after 3 days.

data[2,3]. Our future research goal will focus on the structure determination of the products from the culture broth of S-627 which showed specific activity against dimorphic *Candida*.

Fermentation and isolation

A stock culture of the producing organism S-627 was inoculated into a 500-ml erlenmeyer flask with 100 ml germination medium containing soluble starch 2%, soybean flour 2%, NaCl 0.3%, and $CaCO_3$ 0.3% (pH 7.2 before sterilization). The flasks were cultured at 30℃ for 96 h with an agitation rate of 200 rpm and an aeration rate of 1 vvm. The broth of *Streptomyces* sp. S-627 was extracted with 20 L methanol, and methanol layer was extracted with diaion HP-20 column chromatography eluted with gradient methanol. After confirmation of the activity of the 100%-methanol eluted fraction, we obtained the five-active fractions with silicagel column chromatography using benzene-EtOAc solvent system and preparative TLC (Fig. 1). We designated the fractions as S-627(A), and S-627(B). The UV spectra was as follows : A(222, 367 nm), B(257, 337 nm). The IR spectra (recorded on a IR-470 Shimazu spectrometer) of S-627(A) and (B) were shown in Fig. 2. S-627(A) and (B) show typical IR-absorptions at 742, 1160, 1340, 1460, 1510, 1590, 1650, and 2875 cm^{-1} for S-627(A) and 740, 1010, 1105, 1160, 12

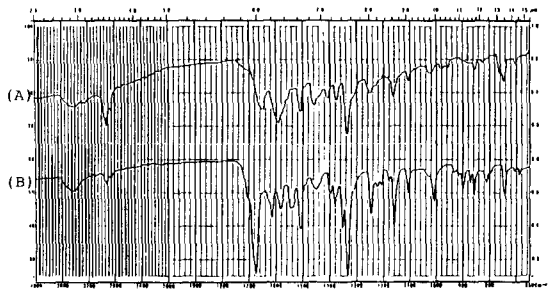

Fig. 2. IR spectra of S-627(A) and (B) on a KBr disc.

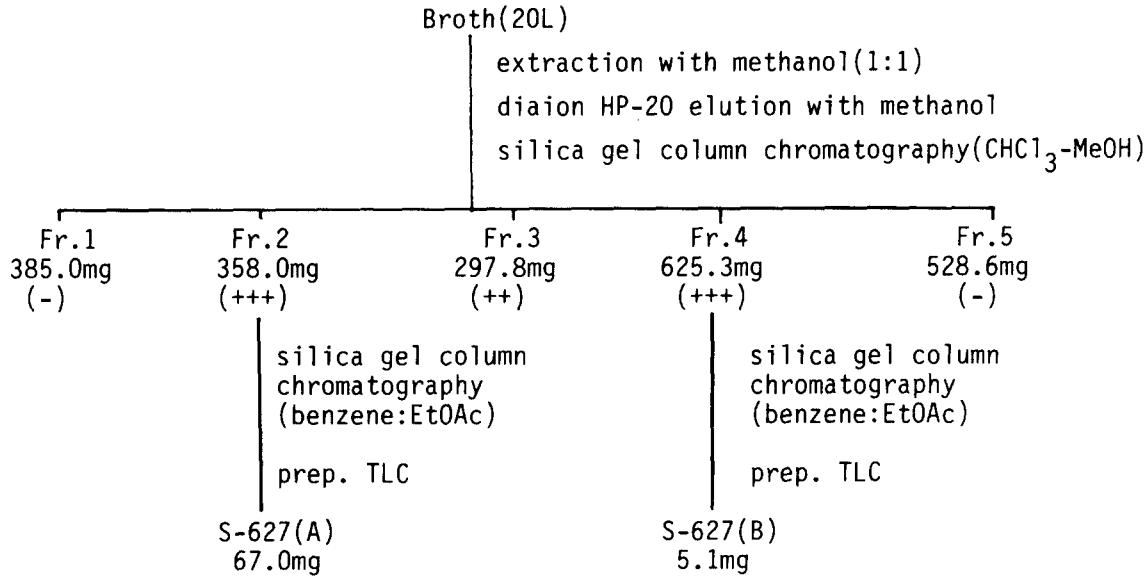

Fig. 1. Isolation procedure of S-627(A) and (B) from the culture broth ofStreptomyces S-627. The activity was shown as follow ; P, no activity ; thediameter of 10P13 mm (+), 14P17 (++), 18P21 (+++), respectively.

45, 1340, 1510, 1540, 1580, 1610, and 1680 cm^{-1} for S-627(B). The structural studies on S-627(A) and (B) are on progress. The complete structure determination will be reported elsewhere.

Biological activities

When each disc contained 50 mg S-627(A) dissolved in 50 ml methanol, it showed 12.0 and 13.5 mm inhibition zone against *Bacillus subtilis* ATCC 27348, and *Micrococcus leuteus* ATCC 9341, respectively. S-627(B) showed an inhibition zone of 15.0 mm at the quantity of 50 μg per disc against *B. subtilis* ATCC 27348.

Characterization of S-627 producer

The aerial mycelia grow abundantly on yeast-malt extract agar. The spores were oval shaped and had a spiny surface. The overall properties of the producer were as follows : sporophore, spiral form ; spores, oval and spiny surface ; the color of mycelia, brown and light black ; the color of aerial mycelia, white and grey ; melanoid pigment, produced ; DAP isomer in cell wall, LL-type. Based on the taxonomic properties, this strain is considered to belong to the genus *Streptomyces*[16].

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초록 : 생리활성물질 S-627(A)와 S-627(B)의 분리

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수정된 선별법(modified selection method)을 사용하여 candiosis에 대한 새로운 생리활성물질(anticandiosis agent)을 생성하는 미생물을 분리하여 *Streptomyces* sp. S-627이라 명명하였으며, 이균의 발효액으로부터 methanol추출, diaion HP-20, silica gel chromatography, 및 분취용 TLC 등의 방법을 사용하여 물리화학적 특성이 다른 S-627(A)와 S-627(B)을 분리하였다.