Korean Journal of Life Science
Vol. 11. No. 5. 483~488, 2001

Physico-Chemical Properties and Antimicrobial Activity of Pyocyanine Produced by *Pseudomonas aeruginosa* KLP-2

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Abstract

The antimicrobial substance produced by *Pseudomonas aeruginosa* KLP-2 strain was purified and identified. The substance was identified as a pyocyanine by the fast atom bombardment mass (FAB-MS). In physico-chemical properties, the pyocyanine was dark blue needles, and was soluble in various organic solvents such as chloroform, methanol, ethanol and ethyl acetate. The pyocyanine possessed a ultraviolet absorbance spectrum in methanol, 0.1 M HCl, and chloroform. The maximum absorption peak of the pyocyanine showed at 318 nm in methanol. The molecular formula of the pyocyanine was determined to be $C_{13}H_{10}N_2O$ and protonated molecular ion species $(M+H)^+$ was observed at *m/z* 211 by FAB-MS. The pyocyanine showed antimicrobial activity against *Bacillus cereus, Bacillus subtilis, Micrococcus luteus, Rodococcus equi, Staphylococcus aureus, Streptococcus faecalis, E. coli, Legionella pneumophila, Shigella flexneri, Shigella boydii, Shigella sonnei,* NAG *Vibrio cholerae, Vibrio parahaemolyticus, Vibrio vulnificus, Yersinia enterocolitica,* and *Saccharomyces cerevisiae.* However, *Salmonella* spp., *Shigella dysenteriae,* 3 strains of *Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Aspergillus niger* were resistant to the pyocyanine. The pyocyanine showed the highest antimicrobial activity aganist *Legionella pneumophila* based on the size of inhibition zone by the disk contained 0.5 μg of the pyocyanine.

Key words – pyocyanine, *Pseudomonas aeruginosa, Legionella*

Introduction

Pseudomonads are bacteria widely distributed in the nature, performing variety of ecological roles in the soil by producing many biologically active secondary metabolites. Among pseudomonads, flourescent *Pseudomonas* produce over 60 secondary metabolites, some of which have antibiotic properties against various microorganisms [10]. Most antimicrobial substances isolated from *Pseudomonas* cultures such as phenazines[2], pyrrolnitrin-type antibiotics, pyo compounds[11], and indole derivatives[10] fall into the class of N-containing heterocycles. The pyocyanine is water-soluble blue-green phenazine pigment produced by *Pseudomonas aeruginosa* and it has antibiotic activity against bacteria[1,11] and fungi [9,12]. Antibiotic action of pyocyanine results from unique redox potential [1,7,8]. Other phenazine compound, phenazine-1-carboxylic acid, has been found to have antifungal activity against *Gaeumannomyces graminis* var. *tritici* and *Candida albicans* [9,13]. The secondary metabolite 2,4-diacetylphloroglucinol (DAPG) produced by *Pseudomonas fluorescens* F113 has been used in the control of potato cyst nematode *Globodera rostochiensis*[4]. 2-Heptyl-4-hydroxypuinoline *N*-oxide produced by *Pseudomonas aeruginosa* showed antibacterial activity against *Staphylococcus aureus* and Gram-positive

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bacteria[11]. Currently, these antibiotic compounds produced from the fluorescent pseudomonads have been characterized chemically and the nature of their antibiotic activities have also been well elucidated[5,10]. In the preliminary study, we screened *Pseudomonas aeruginosa* KLP-2 from cooling tower-waters and the culture broths of *Pseudomonas aeruginosa* KLP-2 showed antimicrobial activity against *Legionella pneumophila, Vibrio cholerae* non O1, *Bacillus cereus, Bacillus subtilis,* and *Staphylococcus aureus.* In this study, we report the purification, identification and antimicrobial spectrum of pyocyanine produced by *Pseudomonas aeruginosa* KLP-2 which considered as an important member of antimicrobial substances.

Materials and methods

Strain

Pseudomonas aeruginosa KLP-2 isolated from a cooling tower-water as described by Park *et al.* (in press, 2001. *Kor. J. Appl. Microbiol. Biotechnol.*), which is a strain producing antimicrobial substance, was used for the production of pyocyanine.

Media and Cultivation for production antimicrobial substance

The strain was grown on Mueller Hinton (MH) agar (Merck) for 24 h at 37℃. Single colony isolated on the agar plate was inoculated into 50㎖ of seed medium in a 250㎖ Erlenmeyer flask and incubated with shaking for 48 h at 35℃. Seed medium contained the following per liter of distilled water: asparagine 3g, K_2HPO_4 1g, $MgSO_4 \cdot 7H_2O$ 0.5g. For main culture, the seed culture was inoculated into 800㎖ of pyocyanine production medium in 4,000㎖ Erlenmeyer flask, then incubated with shaking for 24 h and subsequently without shaking for 4 days at 35℃. The pyocyanine production medium contained the following per liter of distilled water: glycerol 10g, proteose peptone 6g, K_2HPO_4 1g, $MgSO_4 \cdot 7H_2O$ 0.5g. The pH of media were adjusted to 7.0 prior sterilization.

Purification of antimicrobial substance

The purification of pyocyanine produced by *P. aeruginosa* KLP-2 according to a modified procedure described by Baron and Rowe[1] and Fenton *et al.*[5]. After main culture, 500ml of culture broth was centrifuged at 7,000 g for 15 min to remove bacterial cells. The remaining supernatants were extracted in a 0.5 volume of chloroform. The chloroform emulsion was centrifuged at 10,000 g, for 15 min, and the dark-blue chloroform layer was drawn off. The chloroform layer was washed three times with 0.2 volume of distilled water and extracted with 0.33 volume of 0.1 N HCl. The acid layer was washed once with a 0.2 volume of chloroform and titrated with 1.0 M tris (hydroxymethyl) aminomethane-hydrochloride (pH 11.0) until the blue color reappeared. The pigment was extracted exhaustively from this solution with 0.2 volume of chloroform. The resulting dark-blue chloroform layer was loaded on the Sep-Pak® Plus Silica (Waters) cartridge. The antimicrobial substance was eluted with methanol and evaporated to dryness under vaccum. Crystallization of the pyocyanine was taken in the cold from chloroform with the addition of petroleum ether[3].

Thin-layer chromatography and ultra spectroscopy were used to estimate the purity of the pyocyanine preparations. Silica gel thin-layer chromatography (Silica gel 60 F_{254}, Merck) was performed in two different solvent systems, chloroform-methanol (1:1) (system A) and ethyl acetate-acetic acid-water (3:2:1) (system B), as described by Baron *et al.*[1]. Ultraviolet absorption spectrophotometry was obtained in methanol, 0.1 M HCl and chloroform on a Cary 3, UV-Visible Spectrophotometer (Varian). The purified pyocyanine was dissolved in methanol. The solution was applied to a CAPCELL PAK C_{18} column (Shiseido, Type UG 120 5㎛, 4.6×150㎜) for HPLC and developed with acetonitrile-methanol- 0.05 M NaH_2PO_4 containing 5 mM cethyltrimethyl ammonium bromide (15:35:50, v/v/v). The solution was run at a flow rate of 1.5㎖/min and detected at a wavelength of 254 nm.

Chromatography was developed using a Waters 2690 connected to a Waters 996 photodiode array detecter.

FAB-MS spectra was used to confirm the molecular formula of antimicrobial substance produced by *Pseudomonas aeruginosa* KLP-2[9]. Mass spectrometric chromatogram was obtained by JMS-HX110/110A tandom mass spectrometer (JEOL, Tokyo, Japan) using a JMS-DA9000 data system, provided by Korea Basic Science Institute, Taejeon, Korea. All chemicals used in this study were reagent grade or better.

Antimicrobial Activity Assay

Antimicrobial activity against various microorganisms was primarily determined by the paper disk method using 8 mm disk (Toyo, Co., Japan) and the activity was determined with the diameter of clear zone. Twenty-seven strains were used for antimicrobial activity assay. Gram negative and positive strains were obtained from Korea National Institute of Health (KNIH), *Saccharomyces cerevisiae* and *Aspergillus niger* ATCC 9642 were obtained from department of Microbiology in Busan National University. *Legionella pneumophila* ATCC 33152 was cultured on buffered charcol yeast extract agar (BCYE agar, Difco), the other Gram-negative & Gram-positive bacteria were cultured on Muller Hinton agar (Merck). The suspension of microorganisms were prepared in sterile saline and adjusted to 0.5 McFarland standard turbidity. A sterile cotton swab was dipped in the suspension and inoculated by streaking over the surface of BCYE agar and MH agar. *Saccharomyces cerevisiae* and *Aspergillus niger* ATCC9642 were cultured on potato dextrose agar (PDA, Difco) and then spore were harvested with sterilized saline. Spore suspensions (1×10^6spores/㎖) were spread on the PDA plate using a sterile cotton swab. The pyocyanine was prepared in methanol : water (1:24 v/v) and disk containing 0.5㎍ of pyocyanine applied to the surface of agar plates. All plates were incubated 48 h at 35℃. The degree of antimicrobial activity was expressed as the diameter of the inhibition zone.

Results and Discussion

Physico-chemical Properties of antimicrobial substance

The physico-chemical properties of antimicrobial substance produced by *P. aeruginosa* KLP-2 strain were summarized in Table 1. Antimicrobial substance was dark blue needles and was found to be soluble in various organic solvents including chloroform, methanol, ethanol, and ethyl acetate. It was moderately soluble in water and acetone but was insoluble in hexane and diethyl ether. After thin-layer chromatography in solvent system A, a single light-blue spot with an R_f of 0.72 was observed. In solvent system B, a single wine-red spot with an R_f of 0.25 was observed. Purified antimicrobial substance possessed a characteristic ultraviolet absorbance spectrum in methanol, 0.1 M HCl, and chloroform. The maximum absorption peak of antimicrobial substance showed at 318 nm in methanol. This substance possessed the same spectral and spectroscopic properties as pyocyanine[1,9,14].

The antimicrobial substance was analysed on HPLC as a single peak with a retention time of 0.9 min, but

Table 1. Physico-chemical properties of antimicrobial substance produced by *P. aeruginosa* KLP-2 strain

Appearance	Dark blue needles
Absorption maxia (nm)	
in MeOH	238, 318, 713
in 0.1 M HCl	242, 278, 387, 519
in $CHCl_3$	239, 309, 326, 694
Thin-layer chromatograph	
R_f of system A	0.72
R_f of system B	0.25
Solubility	
Souble	Chloroform, methanol, ethanol, ethyl acetate
Moderately soluble	Water, Acetone
Insoluble	Hexane, Diethyl ether
Molecular formula	$C_{13}H_{10}N_2O$
FAB-MS$(M+H)^+$	211

antimicrobial substance showed an extensive tailing effect (Fig. 1). Fernández and Pizarro demonstrated that this tailing effect due to the quaternary nature of the pyocyanine[6].

The molecular formula of antimicrobial substance was determined to be $C_{13}H_{10}N_2O$ and protonated molecular ion species $(M+H)^+$ was observed at m/z 211 by FAB-MS (Fig. 2). As these results, the antimicrobial substance produced by *P. aeruginosa* KLP-2 was identified as a pyocyanine[1,5,9,14]. The average yield of purified pyocyanine was 27㎎/ℓ.

Antimicrobial Activity of purified Pyocyanine

The antimicrobial activity of purified pyocyanine against various microorganisms were shown in Table 2. The pyocyanine showed extensive spectrum of antimicrobial activity; *Bacillus cereus* ATCC 11778, *Bacillus subtilis* ATCC 17686 *Micrococcus luteus* ATCC 10240, *Rodococcus equi* ATCC 6939, *Staphylococcus aureus* ATCC 6538P, *Streptococcus faecalis* ATCC 10541, *E. coli* ATCC 10536, *Legionella pneumophila* ATCC 33152, *Shigella flexneri* ATCC 9473, *Shigella boydii* ATCC 9207, *Shigella sonnei* ATCC 9290, NAG *Vibrio cholerae* ATCC 25872, *Vibrio parahaemolyticus* ATCC 27519, *Vibrio vulnificus* ATCC 33816, *Yersinia*

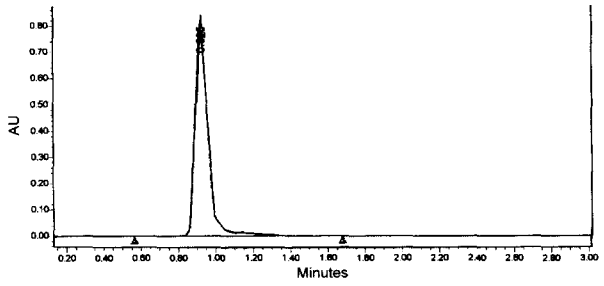

Fig. 1. High performance liquid chromatogram of antimicrobial substance produced by *P. aeruginosa* KLP-2.

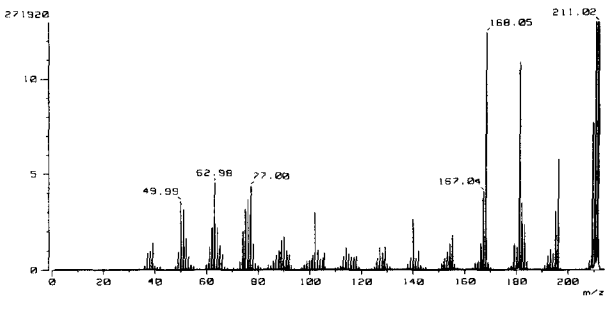

Fig. 2. FAB-MS spectrum of antimicrobial substance produced by *P. aeruginosa* KLP-2.

Table 2. Antimicrobial activity of pyocyanine purified from antimicrobial substance produced by *P. aeruginosa* KLP-2 strain

Microorganisms	Inhibition zone (mm)
Gram positive strains	
Bacillus cereus ATCC11778	16.8
Bacillus subtilis ATCC17686	31.4
Micrococcus luteus ATCC10240	15.1
Rodococcus equi ATCC6939	19.1
Staphylococcus aureus ATCC6538P	16.5
Streptococcus faecalis ATCC10541	12.8
Gram negative strains	
E. coli ATCC9637	10.9
E. coli ATCC10536	11.7
Klebsiella pneumoniae ATCC10031	0
Legionella pneumophila ATCC33152	48.0
Pseudomonas aeruginosa ATCC9027	0
Pseudomonas aeruginosa ATCC27853	0
Pseudomonas aeruginosa KLP-2	0
Salmonella Anatum ATCC9270	0
Salmonella Enteritidis ATCC13076	0
Salmonella Newport ATCC6962	0
Salmonella Paratyphi A ATCC11511	0
Shigella dysenteriae ATCC9752	0
Shigella flexneri ATCC9473	15.5
Shigella boydii ATCC9207	19.7
Shigella sonnei ATCC9290	15.6
NAG *Vibrio cholerae* ATCC25872	22.9
Vibrio parahaemolyticus ATCC27519	20.4
Vibrio vulnificus ATCC33816	17.6
Yersinia enterocolitica ATCC27729	15.6
Yeast	
Saccharomyces cerevisiae	18.0
Mold	
Aspergillus niger ATCC9642	0

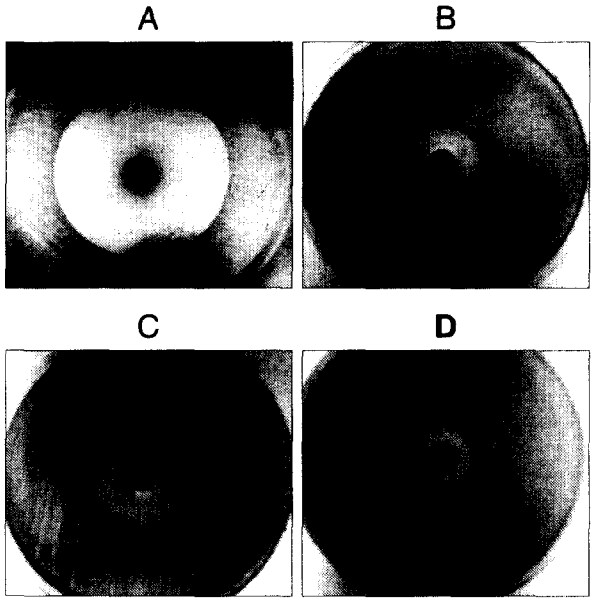

Fig. 3. In vitro inhibition of pyocyanine purified from antimicrobial substance produced by *P. aeruginosa* KLP-2 strain against *Legionella pneumophila* (A), NAG *Vibrio cholerae* (B), *Bacillus subtilis* (C) and *Saccharomyces cerevisiae* (D). Each disk was contained 0.5μg of pyocyanine, and all plates were incubated 48 h at 35℃.

enterocolitica ATCC 27729 and *Saccharomyces cerevisiae* were significantly susceptible. However, *Salmonella* strains, *Shigella dysenteriae* ATCC 9752, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* ATCC 9027, *Pseudomonas aeruginosa* KLP-2, *Klebsiella pneumoniae* ATCC 10031, *E. coli* ATCC 9637, and *Aspergillus niger* ATCC 9642 were resistant to pyocyanine. All of *Pseudomonas aeruginosa* tested, including *Pseudomonas aeruginosa* KLP-2, the producer of pyocyanine, were totally resistant to the level of pyocyanine tested. This result was coincided with the report of Baron and Rowe[1] suggested that resistance of *Pseudomonas* strains to pyocyanine may be characteristic of the entire genus. Baron and Rowe, Chang *et al.*, and Kerr *et al.* have reported that the pyocyanine inhibited much more Gram positive than Gram negative bacteria [1,2,9]. In this study, however, the pyocyanine produced by *P. aeruginosa* KLP-2 strain has been antimicrobial activity not only Gram positive but also Gram negative bacteria. The pyocyanine showed the highest antimicrobial activity aganist *Legionella pneumophila* based on the size of inhibition zone by the disk contained 0.5 μg of the pyocyanine (Fig. 3).

In conclusion, this study indicates that the pyocyanine produced by *P. aeruginosa* KLP-2 strain possessed widely antimicrobial spectrum and antimicrobial activity against *L. pneumophila* was excellent. Therefore, the pyocyanine is recommended as the powerful biocides for control of legionellosis, and application of pyocyanine against *L. pneumophila* is reported here for the first time.

References

1. Baron, S. S. and J. J. Rowe. 1981. Antibiotic action of pyocyanine. *Antimicrob. Agents Chemother.* **20**, 814-820.
2. Brisbane, P. G., L. J. Janik, M. E. Tate and R.F.O. Warren. 1987. Revised structure for the phenazine antibiotic from *Pseudomonas fluorescens* 2-79(NRRL B-15132). *Antimicrob. Agents Chemother.* **31**, 1967-1971.
3. Chang, P. C. and A. C. Blackwood. 1968. Simultaneous production of three phenazine pigments by *Pseudomonas aeruginosa* Mac 436. *Can. J. Microbiol.* **15**, 439-444.
4. Cronin, D., Y. Mo'e nne-Loccoz, A. Fenton, C. Dunne, D. N. Dowling and F. O'Gara. 1997. Role of 2,4-diacetylphloroglucinol in the interaction of the biocontrol Pseudomonad strain F113 with the potato cyst nematode *Globodera rostochiensis*. *Appl. Environ. Microbiol.* **63**, 1357-1361.
5. Fenton, A. M., P. M. Stephens, J. Crowley, M. O'callahaghan and F. O' Gara. 1992. Exploitation of gene(s) involved in 2,4-diacetylphloro- glucinol biosynthesis to confer a new biocontrol capability to a *Pseudomonas* strain. *Appl. Environ. Microbiol.* **58**, 3873-3878.
6. Fermández, R. O. and R. A. Pizarro. 1997. High-performance liquid chromatograph analysis of *Pseudomonas aeruginosa* phenazines. *J. Chromatog. A.* **771**, 99-104.
7. Hassan, H. M. and I. Fridovich. 1980. Mechanism of the antibiotic action of pyocyanine. *J. Bacteriol.* **141**, 156-163.

8. Hassett, D. J., L. Charniga, K. Bean, D. E. Ohman and M. S. Cohen. 1992. Response of *Pseudomonas aeruginosa* to pyocyanine: mechanism of resistance, antioxidant defense, and demonstration of a manganese- cofactored superoxide dismutase. *Infect. Immun.* **60**, 328-336.
9. Kerr. J. R., G. W. Taylor, A. Ruman, N. Høiby, P. J. Cole and R. Wilson. 1999. *Pseudomonas aeruginosa* pyocyanin and 1-hydroxyphe- nazine inhibit fungal growth. *J. Clin. Pathol.* **52**, 385-387.
10. Leisinger, T. and R. Margraff. 1979. Secondary metabolites of the fluorescent pseudomonads. *Microbiological Reviews.* **43**, 422-442.
11. Machan, Z. A., G. W. Taylor, T. L. Pitt, P. J. Cole and R. Wilson. 1992. 2-heptyl-4-hydroxyquinolin N-oxide, an antistaphylococcal agent produced by *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* **30**, 615-623.
12. Min, B. Y., J. Y. Shim, K. W. Kim, J. K. Lee, H. T. Choi and K. S. Yoon. 1996. Fungal-sporulation suppressing substances produced by *Pseudomonas aeruginosa* KMCS-1. *J. Microbiol.* **34**, 284-288.
13. Thomashow, L. S. and D. M. Weller. 1988. Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*. *J. Bacteriol.* **170**, 3499-3508.
14. Waston, D., J. MacDermot, R. Wilson, P. J. Cole and G. W. Taylor. 1986. Purification and structural analysis of pyocyanin and 1-hydroxyphenazine. *Eur. J. Bio Chem.* **159**, 309-313.

(Received July 23, 2001; Accepted September 4, 2001)

초록 : *Pseudomonas aeruginosa* KLP-2가 생산한 Pyocyanine의 항균활성 및 생리화학적 성상

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최근 다양한 분야에서 생물학적 제제개발에 관한 연구가 활발하다. 본 연구에서는 생물학적 제제개발을 위한 일련의 연구로 *Pseudomonas aeruginosa* KLP-2가 생산하는 항균성 물질을 동정하고, 생리화학적 특성을 확인하였다. 본 항균성 물질을 정제하여 FAB-MS로 확인한 결과 pyocyanine으로 동정되었으며, 진청색의 needles 성상을 나타내었고 chloroform, methanol, ethyl acetate와 같은 용매에 높은 용해도를 나타내었다. Pyocyanine의 UV spectrum은 methanol에서 최대 흡광치 (318 nm)를 보였고, FAB-MS로 확인한 결과 *m/z* 211에서 protonated molecular ion $(M+H)^+$이 관찰되었으며, 분자식은 $C_{13}H_{10}N_2O$로 확인되었다. Pyocyanine은 *Bacillus cereus, Bacillus subtilis, Micrococcus luteus, Rodococcus equi, Staphylococcus aureus, Streptococcus faecalis, E. coli, Legionella pneumophila, Shigella flexneri, Shigella boydii, Shigella sonnei*, NAG *Vibrio cholerae, Vibrio parahaemolyticus, Vibrio vulnificus, Yersinia enterocolitica* 및 *Saccharomyces cerevisiae* 등의 다양한 세균에 대하여 항균활성을 보여 비교적 넓은 항균범위를 나타내었으며, 특히 *Legionella pneumophila*에 대하여 0.5㎍의 pyocyanine을 함유한 disk가 48㎜의 억제환을 나타내어 가장 높은 항균활성을 보였다.