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Purification and Characterization of Anti-*Vibrio* Substances from Marine *Pseudoalteromonas* sp. A1-J11

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***Key words*:** anti-*Vibrio* substance, *Pseudoalteromonas*, UV absorption spectrum, HPLC, mass spectrometry

Abstract

Pseudoalteromonas sp. A1-J11 isolated from coastal seawater of Kagoshima Bay, Kagoshima Prefecture, Japan was found to produce anti-*Vibrio* substances extracellularly. Anti-*Vibrio* substances were extracted from the culture supernatant of A1-J11 with ethyl acetate and isolated by using a hydrophobic column chromatography (Sephadex LH-20), thin layer chromatography (Silica gel), and HPLC (Mightysil RP-18 GP Aqua). Isolated fractions, Fr-1, Fr-2, and Fr-3 from Sephadex LH-20 column chromatography showed similar UV absorption spectra with λ_{max} at 210-235, 315, and 327 nm in methanol and growth inhibitory activity against *Vibrio harveyi* strain. AVS-2, the major purified substance obtained from Fr-2 by TLC and HPLC showed a molecular weight of 215 as determined by mass spectrometry.

Vibrio harveyi is a halophilic Gram-negative species of which the major isolates appear non-pathogenic but some strains cause luminous vibriosis to marine fish and shrimp.[1-5)] In disease control, many antibiotics have been used to bring about serious problem associated with drug resistance. The probiotic application of effective bacteria is expected to prevent disease of aquaculture animals.[6-9)] For example, a *Bacillus* strain was reported to be used as a probiotic administrated to larvae of black tiger shrimp. Siderophore-producing *Pseudomonas fluorescens* has been found to suppress the mortality of rainbow trout infected with *V. anguillarum*.[8)] Recently, this technique has been gaining popularity with an increase in the use of probiotic bacteria. For this purpose, many bacteria with the potential as biocontrol agents have been identified, and several biologically active substances have been isolated from marine bacteria.[10-13)]

The authors reported isolation of marine bacteria possessing antibacterial activity against *Vibrio* species including *V. harveyi* in the previous paper. In this work, the authors purified an anti-*Vibrio* substance from culture supernatant of marine *Pseudoalteromonas* A1-J11 using TLC and HPLC methods, and determined UV absorption spectrum and molecular mass of the substance.

Materials and Methods

Bacterial strains and media

Antagonistic strain A1-J11 (*Pseudoalteromonas*

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sp.) was isolated from sea water in Kagoshima Bay, Kagoshima Prefecture, Japan as described in the previous paper.[14] *Vibrio harveyi* strain ATCC 14126 was obtained from the American Type Culture Collection, USA (ATCC).

Bacterial strains were cultured in a modified ZoBell medium (Z-CII)[15] containing Polypepton (Nippon Seiyaku, Tokyo, Japan) 5 g/l and yeast extract (Nippon Seiyaku) 1g/l in 3/4 strength artificial seawater (ASW, Herbst's formula composed of NaCl 30.0 g, KCl 7.0 g, $MgCl_2 \cdot 6H_2O$ 10.8 g, $MgSO_4 \cdot 7H_2O$ 5.4 g, and $CaCl_2 \cdot 2H_2O$ 1.0 g per l). The bacterial strains were stored at -80°C in Z-CII containing 20% glycerol and subcultured into Z-CII agar (1.5%) slants and maintained at 4°C. Target strain, *V. harveyi* ATCC 14126 used for disk diffusion assay were grown in 10 ml of Z-CII broth in a rotating L-shaped test tube at 25°C for 2 days.

Organic solvent extraction of active substances

The supernatant of 4 day culture (300 ml x 4 flasks) of strain A1-J11 was separated by centrifugation at 10,000 x g for 15 min and extracted with an equal volume of ethyl acetate (EtAc). Ethyl acetate fraction was evaporated and residual materials obtained were dissolved in 30 ml of deionized water (DW). The water solution was extracted with an equal volume of chloroform ($CHCl_3$). The $CHCl_3$ fraction was evaporated and residual materials were dissolved in small volume (2-3 ml) of methanol (MeOH).

Purification of anti-*Vibrio* substances from strain A1-J11

The $CHCl_3$ extract was eluted through a hydrophobic column of Sephadex LH-20 (Amersham Biosciences, 25 mm x 300 mm) using a mobile phase of MeOH:DW (3:7, v/v). Each 10 ml eluate in fraction tubes was determined for absorbance at 210 nm and 325 nm using a spectrophotometer (Hitachi, U-2010) and tested for inhibitory activity by disk diffusion assay. The pooled active fractions from the column chromatography were concentrated and applied to thin-layer chromatography (TLC), which was developed with a mobile phase of $CHCl_3$:EtAc:acetone (5:1:3, v/v). A TLC plate, after developing, was exposed to a UV lamp (275 nm) and visible and UV absorbed areas on the TLC plate were removed and extracted with acetone. The acetone was evaporated and redissolved in small volume (1-3 ml) of MeOH. The pooled active fractions from TLC plates were concentrated and applied to RP-HPLC (Mightysil RP-18 GP Aqua 250-4.6, Kanto Chemicals, 4.6 mm I.D. x 250 mm) and developed using 25% acetonitrile/75% DW eluent solution at a flow rate of 0.6 ml/min with monitoring at 325 nm. Collection of purified fractions was performed using large column of Mightysil RP-18 (10 x 250 mm) with 20% acetonitrile/80% DW as eluting solution.

Inhibitory assay

Inhibitory activity against a test bacterium was conducted by disk diffusion assay in which the test substances were dissolved in methanol (MeOH) onto a paper disk (8 mm, diameter), dried, and applied to a double-layer Z-CII agar plate (1.5% agar bottom layer/0.5% agar top layer seeded with the bacterial culture). Inhibitory activity was determined by subtracting the radius of the paper disk from that of the inhibition zone on the double-layer plate.

Molecular mass spectrometry

The purified substance was dissolved in MeOH and applied to a mass spectrometer with electrospray ionization (MAT 900XL, Finnigan and API 2000

LC/MS/MS System, Applied Boisystemes).

Results

Separation of active fractions by Sephadex LH-20 column

The culture supernatant of strain A1-J11 was extracted with EtAc and residual materials were redissolved in MeOH. The crude extract in MeOH was applied to an open column of Sephadex LH-20 (25 x 300 mm). As shown in Fig. 1, 4 peaks with absorption at 325 nm and 1 peak at 410 nm (yellow colored) appeared in the column chromatography. Each fraction (Fr-1, 2, and 3) detected at 325 nm possessed UV absorption spectrum as shown in Figs. 2, 3, and 4, respectively. These absorption spectra were very similar in absorption profile with 210-235, 315, and 327 nm as absorption maxima. Inhibitory activity against *V. harveyi* ATCC 14126 was strongest in Fr-2 than those in Fr-1 and Fr-3 (Fig. 5).

Separation of active fractions by TLC

A TLC plate applied with Fr-2 from Sephadex LH-20 column chromatography was developed and exposed to a UV lamp. Three fractions on TLC plate indicated in Fig. 6 were extracted with acetone and redissolved in MeOH. Fig 7 shows UV absorption spectrum of Fr-2-b in MeOH.

Purification of active substance by HPLC

A fraction, Fr-2-b obtained from TLC plate was applied to a RP-HPLC column (Mightysil RP-18 GP Aqua). As shown in Fig. 8, one minor and one major fraction (designated as AVS-2) were eluted at 120.1 and 130.3 min, respectively. Both fractions demonstrated inhibitory activity against *V. harveyi* ATCC 14126 (Fig. 9)

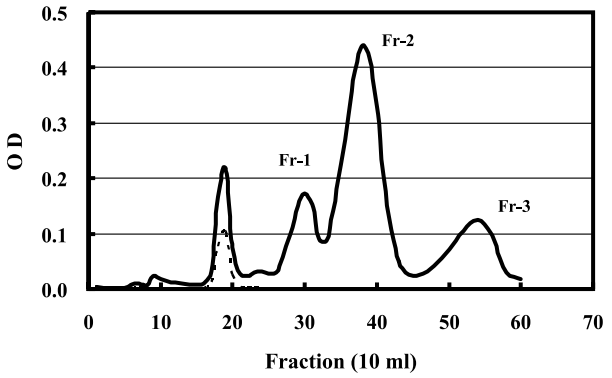

Fig. 1. A column chromatography of crude extract over Sephadex LH-20 eluted with MeOH/DW(3:7, v/v). Straight line indicates absorbance at 325 nm and dotted line indicates absorbance at 410 nm.

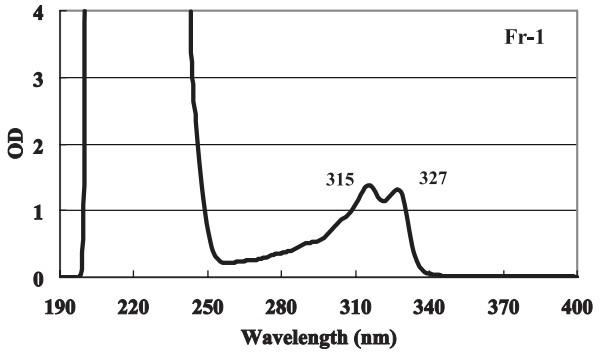

Fig. 2. UV absorption spectrum of Fr-1 on Sephadex LH-20 column chromatography.

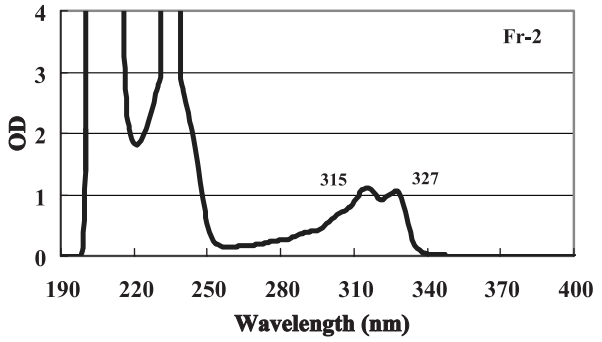

Fig. 3. UV absorption spectrum of Fr-2 on Sephadex LH-20 column chromatography.

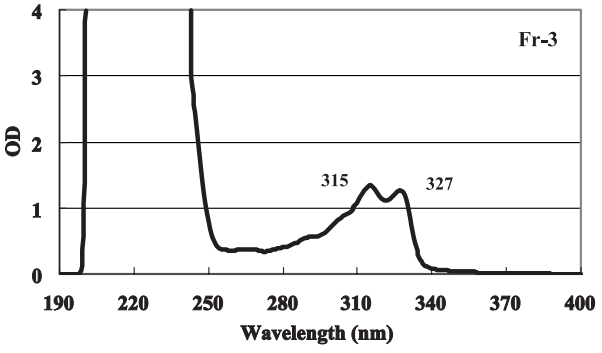

Fig. 4. UV absorption spectrum of Fr-3 on Sephadex LH-20 column chromatography.

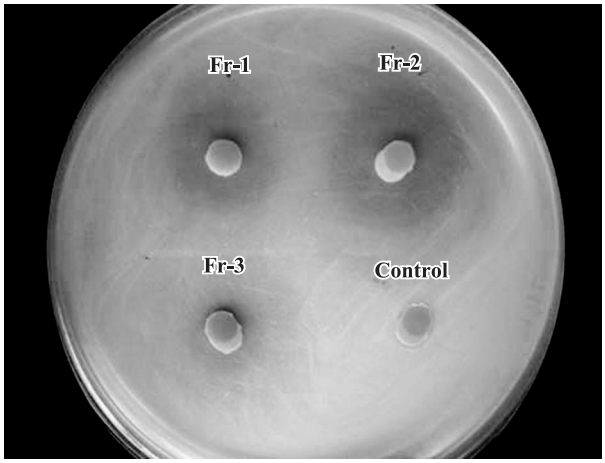

Fig. 5. Growth inhibitory activity of active fractions separated by Sephadex LH-20 column chromatography on a double layer agar plate containing *V. harveyi* ATCC 14126.

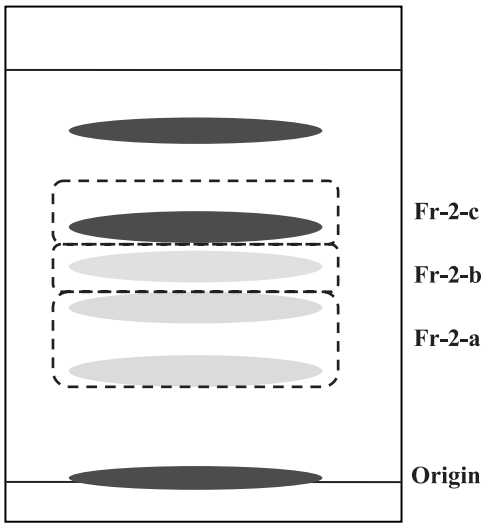

Fig. 6. Thin layer chromatography on silica gel of Fr-2 obtained after Sephadex LH-20 column chromatography. The TLC plate after developing was exposed to a UV lamp.

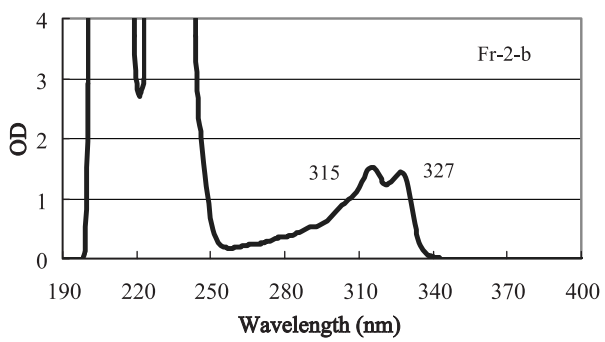

Fig. 7. UV absorption spectrum of Fr-2-b on thin layer chromatography.

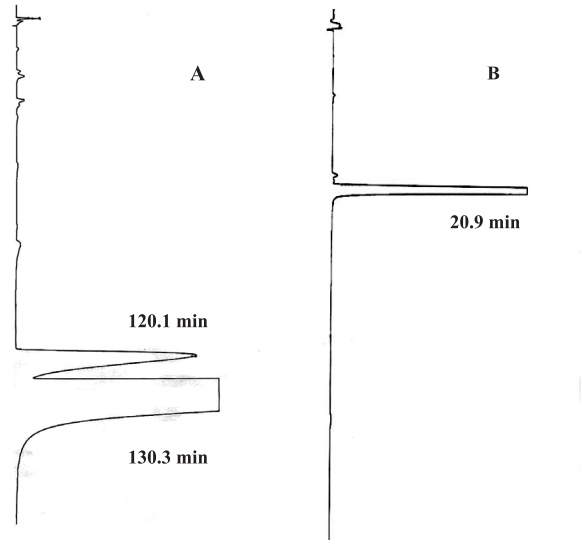

Fig. 8. High performance liquid chromatography of Fr-2-b. A, HPLC of Fr-2-b on a column packed with Mightysil RP-18 GP (250 x 10 mm), 20% acetonitrile/80% DW as mobile phase; B, HPLC of the major fraction of Fr-2-b on a column with Mightysil RP-18 GP (250 x 4.6 mm), 25% acetonitrile/75% DW as mobile phase.

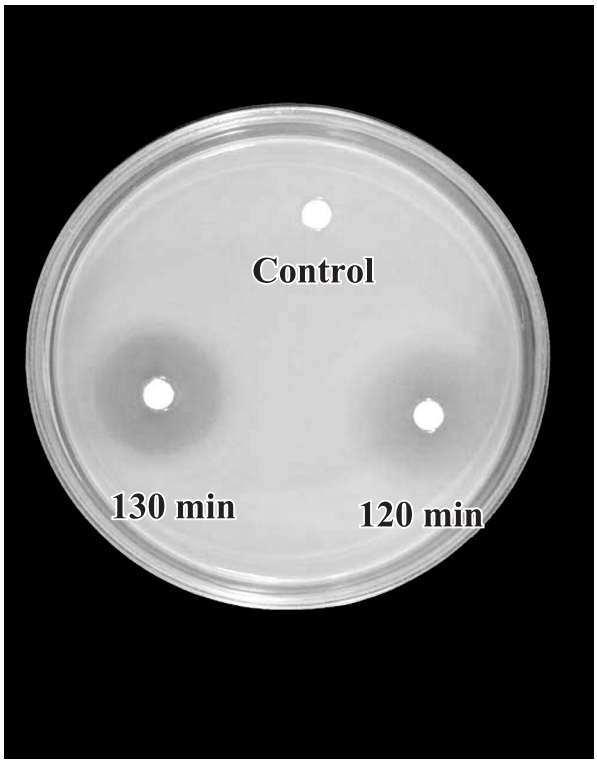

Fig. 9. Growth inhibitory activity of active fractions separated by HPLC on a double layer agar plate containing *V. harveyi* ATCC 14126. 120 min and 130 min indicate the fractions eluted at 120.1 min and 130.3 min on HPLC, respectively.

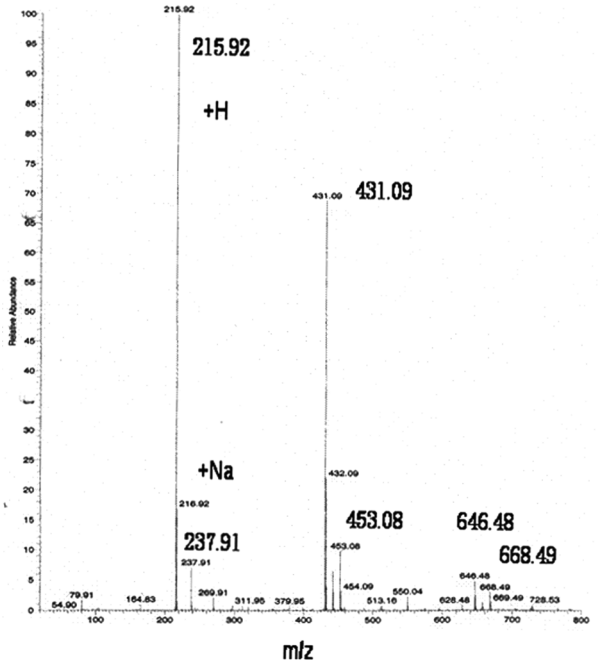

Fig. 10. Low resolution mass spectrum of the purified substance, AVS-2 recorded on a Finnigan MAT 900XL mass spectrometer.

Molecular mass spectrometry

Fig. 10 demonstrated molecular mass signals in mass spectrometry of a purified substance (AVS-2) obtained by HPLC. Three paired signals (m/z) of 215.92 and 237.91, 431.09 and 453.08, and 646.48 and 668.49 (m/z) were detected in mass spectrometry. The signal of 215.92 indicates the molecular mass of AVS-2 plus 1.0 of proton; that of 237.91 indicates AVS-2 plus 23.0 of Na. The signals of 431.09 and 646.48 were suggested to be from doublet and triplet molecules, respectively.

Discussion

Since the discovery of penicillin in 1929, many bacteria and fungi have shown to produce bioactive substances including antibiotics. Especially, the biological activities of secondary metabolites produced by pseudomonads have been investigated from the beginning of the 20th century.[16, 17] Most antibiotics isolated from *Pseudomonas* spp. were found to comprise phenazines, pyrrolnitrin-type antibiotics, pyo compounds (pseudanes), pterines, and indole derivatives.[18] In 1945, Hayes *et al*. obtained antibiotic compounds, pyo compounds from ethanol extract of *P. aeruginosa* cells.[19] Pyo compounds were also detected in a marine pseudomonad.[20] Among pseudomonads phenazine pigments are produced by the fluorescent pseudomonads, in particular with *P. aeruginosa*, which produces the blue pigment pyocyanine.[21] Pyrrolnitrin, an antifungal antibiotic produced by many fluorescent and nonfluorescent *Pseudomonas* spp. is known to be synthesized in several steps from tryptophan.[22]

The authors isolated antibitotic substances against *V. harveyi* from the culture supernatant of a marine *Pseudoaltermonas* strain. These compounds showed very similar UV absorption spectra with λ_{max} at 210-235, 315, and 327 nm in methanol. A major compound, AVS-2 was found to have a molecular weight of 215 as determined by mass spectrometry. In UV absorption spectra of isolated compounds, λ_{max} at 210-235, 315, and 327 nm suggest that these compounds consist of a polycyclic aromatic hydrocarbon structure, like pyo compounds produced by *Pseudomonas* spp. Further research is planned to clarify the chemical structure of isolated compounds by using of mass spectrometry and NMR analysis.

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