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Antibiotic Production by Marine Pigmented Bacteria— II

Purification and Characterization of Antibiotic Substance of *Alteromonas luteoviolacea*

Taizo SAKATA[*1], Kunio SAKAGUCHI[*1] and Daiichi KAKIMOTO[*2]

Abstract

A marine purple bacterium, *Alteromonas luteoviolacea* 9K-V9 isolated from sea water of Kinko Bay, Japan produced proteinous antibiotic substances. The crude extract from the cells contained two major components, lower molecular and higher molecular ones responsible for the antibiotic and autotoxic activities. The higher molecular component may composed of the lower molecular component and other cellular materials.

The lower molecular substance was purified by using DEAE-sephadex A-50 chromatography, Toyopearl HW-55 gel filtration and polyacrylamide gel electrophoresis. The purified substance was shown to be heat-labile but relatively stable to a wide range of pH and to have an approximate molecular weight of 50,000.

Marine purple bacteria identified as the species, *Alteromonas luteoviolacea* were previously reported to produce antibiotic substance(s) which inhibited the growth of various kinds of bacteria.[1-3] The crude extract derived from marine purple bacteria were simultaneously shown to the self inhibition against these bacteria. The antibiotic activity was found in the crude extract obtained from the cells by sonication and the culture filtrate, and shown to be due to heat-labile macromolecular substance(s).[2]

A macromolecular antibiotic produced by marine purple bacteria has been demonstrated by GAUTHIER.[4,5] This antibiotic substance was shown to be polyanionic polysaccharide and to act at the level of the respiratory chain by setting up of a flavinic respiration. ANDERSON *et al*[6] also have reported that various brominated antibiotics were produced by marine *Chromobacterium* strains. Some of brominated compounds were found to be responsible for antoinhibition of the *Chromobacterium* itself as well as for antibiotic action against other bacteria.

The purpose of this paper has been to investigate the purification and characterization of the macromolecular antibiotic substances of a marine purple bacterium, *Alteromonas luteoviolacea* strain 9K-V9.

*1 鹿児島大学水産学部微生物研究室 (Laboratory of Microbiology, Faculty of Fisheries, Kagoshima University, 50-20 Shimoarata 4, Kagoshima, 890 Japan)
*2 Professor emeritus of Kagoshima University.

Materials and Methods

Organisms and growth conditions *Alteromonas luteoviolacea* strain 9K-9V was isolated from the coastal water of Kinko Bay, Kagoshima Prefecture, Japan. The strain was grown on modified ZoBell 2216E (ZS medium) agar slant at 25℃ and subcultured every four days. The organism was inoculated into 5 l flasks containing 3 l of ZS broth. Incubation was carried out statically at 25℃ for the production of antibiotic substance. *A. luteoviolacea* 9K-V9 and *Vibrio parahaemolyticus* HK-15 (serotype O 5 : K15, obtained from Dr. K. TAKAGI at Hokkaido University) were used for the examination of antibiotic susceptibility.

Antibiotic activity Growth inhibition of test organisms was examined by the double agar method described previously.[2] An aliqout (0.1 ml) of actively grown cell culture was mixed with soft agar medium and overlayered on ZS agar (pH 6.3) and Trypticase soy agar (TSA, pH 6.3) for *A. luteoviolacea* 9K-V9 and *V. parahaemolyticus* HK-15, respectively. The paper discs impregnated with the cell extract or polyacrylamide gel slices after electrophoresis, were placed on the double layer agar plates of test organisms. Inoculated agar plates were incubated at 25℃ for 48 hr and then zone of growth inhibition were measured.

Preparation of crude extract Three liters of modified ZS broth were inoculated with *A. luteoviolacea* 9K-V9 and incubated for 3 days at 25℃. The cells were harvested by centrifugation at 10,000 x g for 20 min at 4℃. The cells were then suspended in 30 ml of 0.05 M Tris-HCl buffer (pH 7.6) and sonicated for 5 min, followed by centrifugation at 17,000 x g for 20 min at 4℃. The supernatant solution was mixed with an equal volume of cold acetone and kept until a turbid suspension was obtained. The pellet obtained by centrifugation at 17,000 x g for 20 min at 4℃ was dissolved in 10 ml of 0.05 M Tris-HCl buffer (pH 7.6) and dialysed against the same buffer overnight. Antibiotic activity of the crude extract was measured against *A. luteoviolacea* 9K-9V and *V. parahaemolyticus* HK-15 by using the double layer-paper disc method.

Purification of antibiotic substance

a) **Ion exchange chromatography** Ten ml of the crude extract were loaded onto DEAE-sephadex A-50 column (1.5 x 25 cm) which had been equilibrated previously with 0.05 M Tris-HCl buffer (pH 7.6).

Fractions (7 ml, each tube) were eluted by using a salt gradient (0-1.5 M) in 0.05 M Tris-HCl buffer with a flow rate of 23 ml per hr. The protein content of each tube was determined by measuring the absorbance at 280 nm.

b) **Gel filtration** Antibiotic Fractions A and B which were obtained by ion exchange chromatography, were concentrated by dialysis against granulated sugar. Six ml of each concentrated fractions were applied individually to a Toyopearl HW-55 (superfine, Toyosoda co.) column (2.6 x 90 cm). Fractions (3.5 ml) were eluted with 0.05 M Tris-HCl buffer (pH 7.6) at a flow rate of 10 ml per hr.

c) **Polyacrylamide gel electrophoresis (PAGE)** Antibiotic fractions from each step of purification were subjected to disc electrophoresis (7.5 % polyacrylamide gel) according to the method of DAVIS.[7] Electrophoresis was carried out at 2.5 mA per gel. After electrophoresis, one pair of the gels for same sample was stained with Coomassie Brilliant

Blue R250 (Nakarai chemicals) in order to detect protein bands. Another one was sliced into small segments (0.5 cm in length), that were placed on a inoculated double layer plate to detect antibiotic activity. The molecular wight of antibiotic fractions were determined by using SDS-PAGE (7.5 % acrylamide slab gel containing 0.1 % SDS) as described by LAEMMLI.[8] Oligomer proteins of horse cytochrome C (Oriental Yeast Co.) were used for the calibration of molecular weight as marker proteins.

Effect of pH, temperature and divalent cation on antibiotic stability To examine the effect of pH on antibiotic stability, Fraction A solutions were dialyzed for 48 hr at 10℃ against the following buffers; 0.1 M HCl (pH 1.0), 0.05 M citrate buffer (pH 3.0-5.0), 0.05 M Tris-HCl buffer (pH 7.6-9.0), 0.05 M carbonate buffer (pH 11.0) and 0.1 M NaOH (pH 13.0). The remaining activity of each solution was measured after dialyzation against 0.05 M Tris-HCl buffer (pH 7.6) for 24 hr.

Results

Purification of antibiotic substance The crude extract from cells were subjected to DEAE-sephadex A-50 anion exchange chromatography. Most of the proteins that lack antibiotic activity were passed through a DEAE-sephadex A-50 column. Antibiotic activities were separated into two fractions at NaCl concentrations between 0.5 and 0.7 M (Fraction A) and above 0.8 M (Fraction B) as shown in Fig. 1. The activity of Fraction B was eluted as a broad active peak. Therefore, Fraction A was chosen for further purification in this study. In the first gel filtration on Toyopearl HW-55, antibiotic activity was associated with the second peak of proteins (Fig. 2). When the pool of tube fractions with activity was subjected to the second gel filtration on Toyopearl HW-55, antibiotic activity coincided with the single peak of absorbance at 280 nm (Fig. 3).

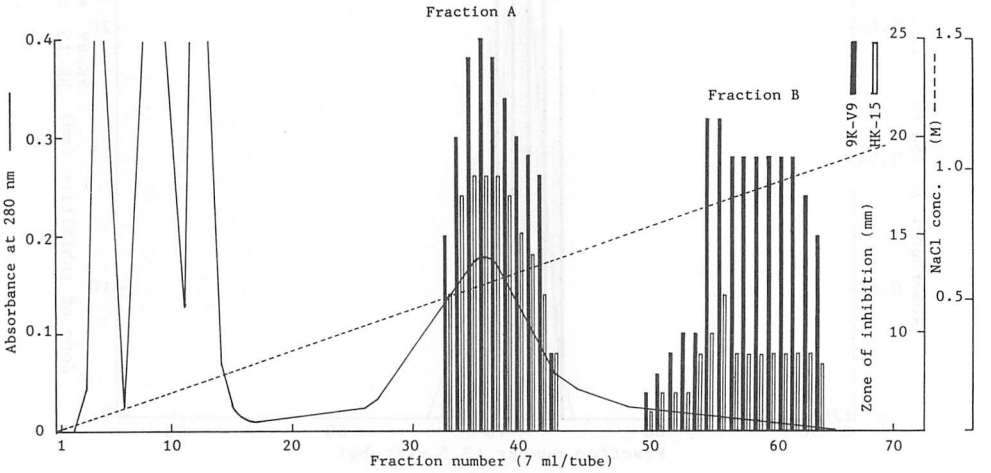

Fig. 1. DEAE Sphadex A-50 column chromatography of the antibiotic substance. The crude extract was applied to a column (1.5 x 25cm) in 0.05 M Tris-HCl buffer (pH 7.6). The retained substances were eluted with a linear gradient of 0 to 1.5 M NaCl in the same buffer.

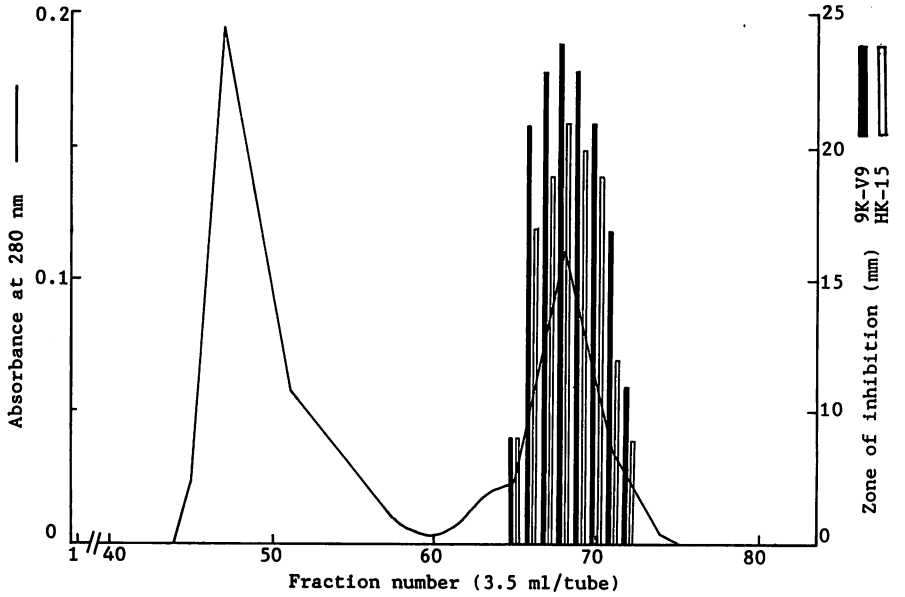

Fig. 2. Gel filtration of the antibiotic substance on Toyopearl HW-55. Fraction A from DEAE Sephadex A-50 chromatography was applied to a column (2.6 x 90cm) which was developed with 0.05 M Tris-HCl buffer (pH 7.6).

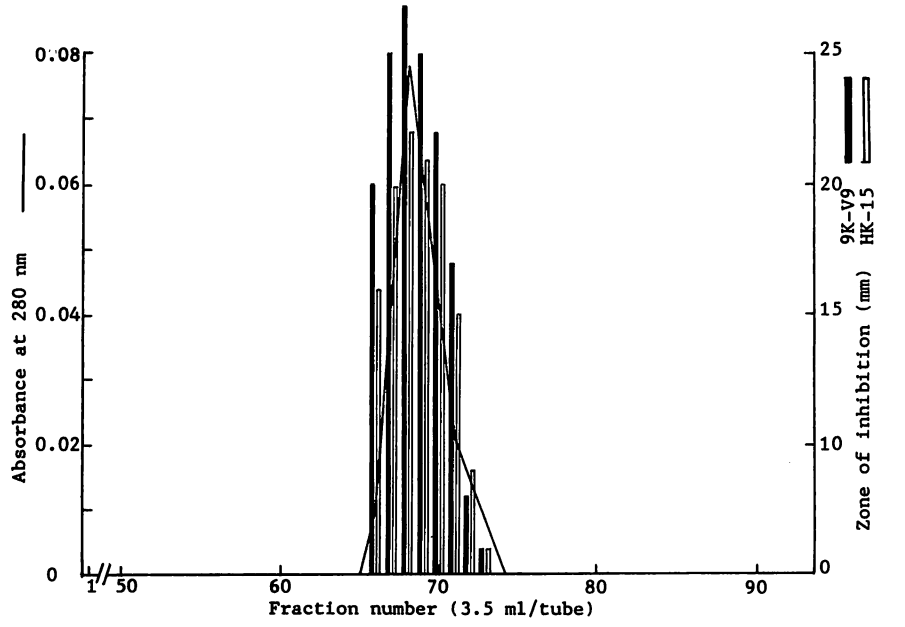

Fig. 3. Second Toyopearl HW-55 gel filtration of the antibiotic substance. The active fraction from first gel filtration was subjected to second gel filtration on Toyopearl HW-55 column (2.6 x 90cm)

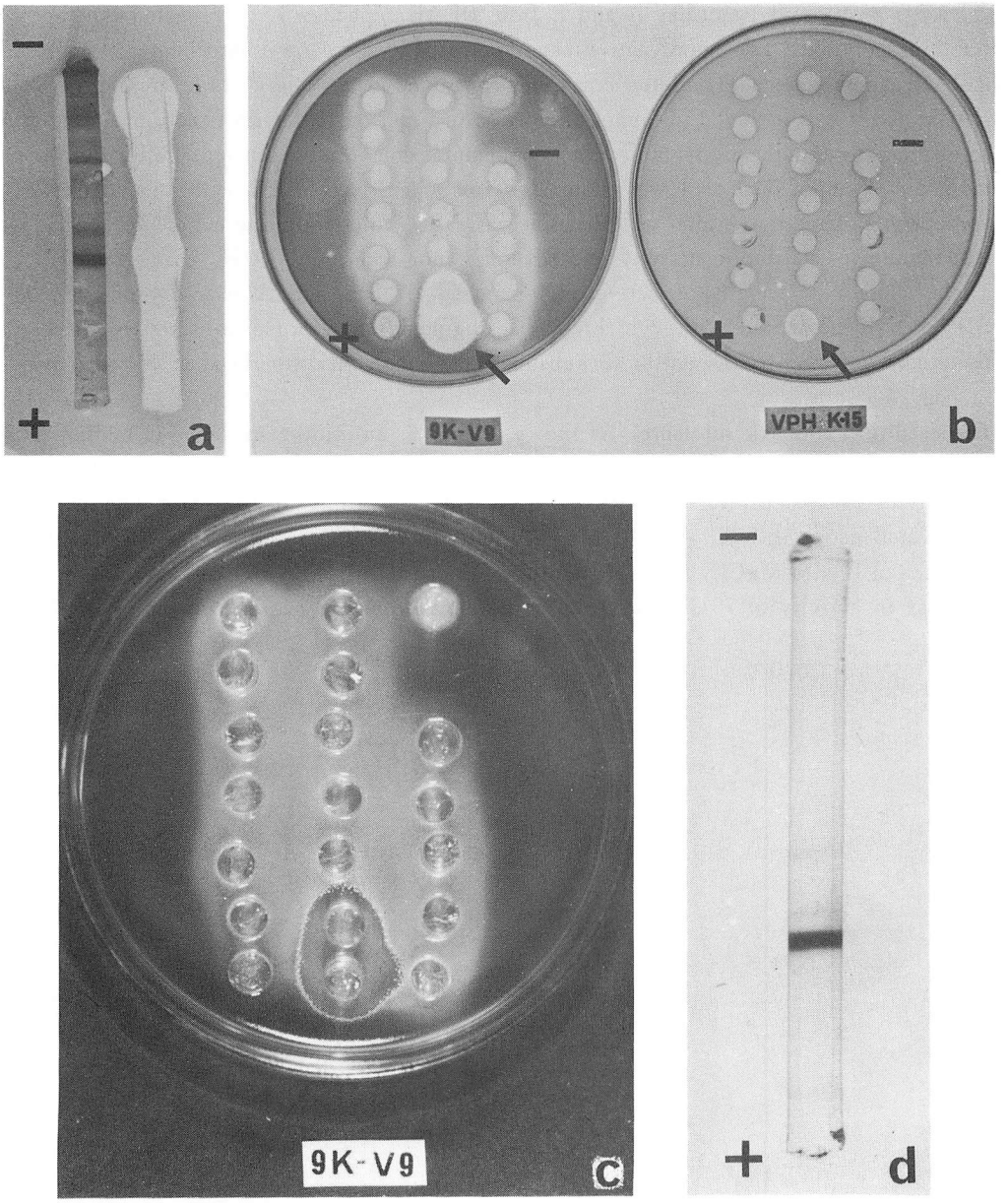

Fig. 4. PAGE of the antibiotic substance from *A. luteoviolacea* 9K-V9. Migration from top (−) to bottom (+).

(a) The crude extract. Left; a gel stained with Coomassie Brilliant Blue, right; a gel incubated on the double agar layer of *V. parahaemolyticus* HK-15.

(b) Fraction A after second Toyopearl HW-55 gel filtration. Left; the gel slices incubated on the lawn of 9K-V9, right; the gel slices incubated on the lawn of HK-15.

(c) The gel slices of Fraction A incubated on the lawn of 9K-V9.

(d) A gel of Fraction A stained with Coomassie Brilliant Blue.

PAGE profiles of Fractions A and B from DEAE-sephadex A-50 revealed that both fractions contained two major active bands corresponded to lower molecuar and higher molecular components. The latter component remained at the origin of the running gel of PAGE and it was included in higher proportion in Fraction B than in Fraction A. These results suggest that the higher molecular component is composed of the aggregation of lower molecular components or lower molecular component associated with other cellular components. Fraction A after gel filtration was shown to contain one major protein band responsible for antibiotic activity and several minor bands on PAGE (Fig. 4).

The molecular weight of active protein band of Fraction A was determined on SDS-PAGE compared with cytochrome C oligomers from horse as standard marker proteins. On SDS-PAGE, antibiotic substance had a molecular weight of 50, 000 as shown in Fig. 5.

The stability of antibiotic substance As shown in Fig. 6, antibiotic substance (Fraction A) was quite stable between pH 1. 0 and 11. 0, and remained fairly stable to retain more than 70 % activity even at pH 13. Activity was stable at temperature up to 57℃ but gradually inactivated above at 57℃ after 10 min. All activity was lost in 10 min at 70℃. In the presence of 10 mM $MgCl_2$, the heat stability of the antibiotic activity was enhanced, i. e. the loss in activity at 70℃ was 50 % of the activity before heat treatment (Fig. 7).

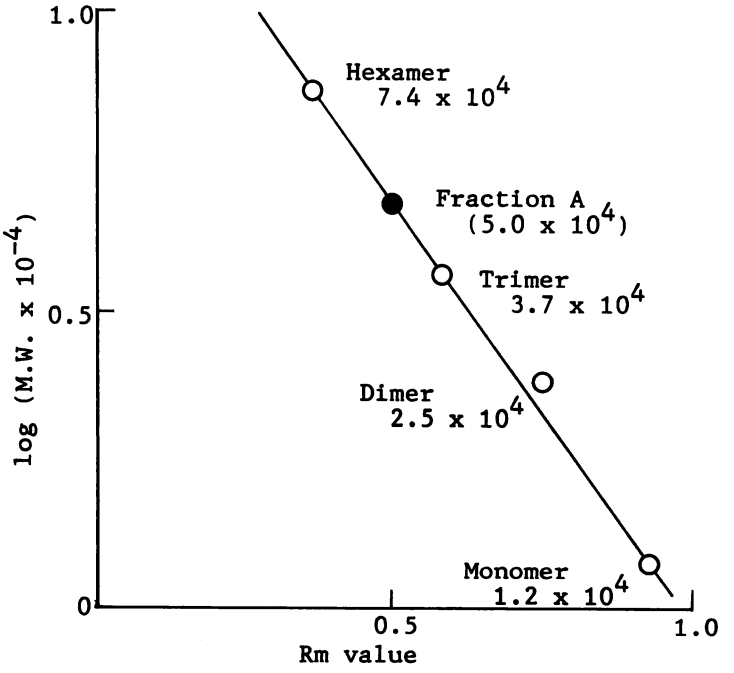

Fig. 5. Molecular weight of the antibiotic substance by SDS-polyacrylamide gel electrophoresis. The migration of MW-Marker proteins (Cytochrome C of horse) was plotted against molecular weight.

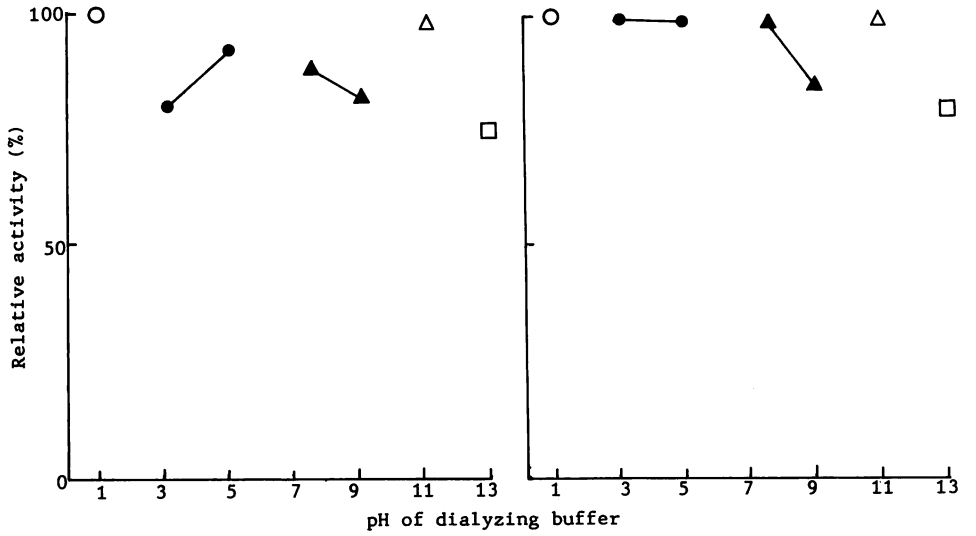

Fig. 6. Effect of pH on the stability of antibiotic substance.
Fraction A after gel filtration was dialyzed for 48 hr against the following buffer; ○ 0.1 M HCl, ● 0.05 M citrate buffer, ▲ 0.05 M Tris-HCl buffer, △ 0.05 M carbonate buffer, □ 0.1 M NaOH. The remaining activity was measured for the strains 9K-V9 (left) and HK-15 (right).

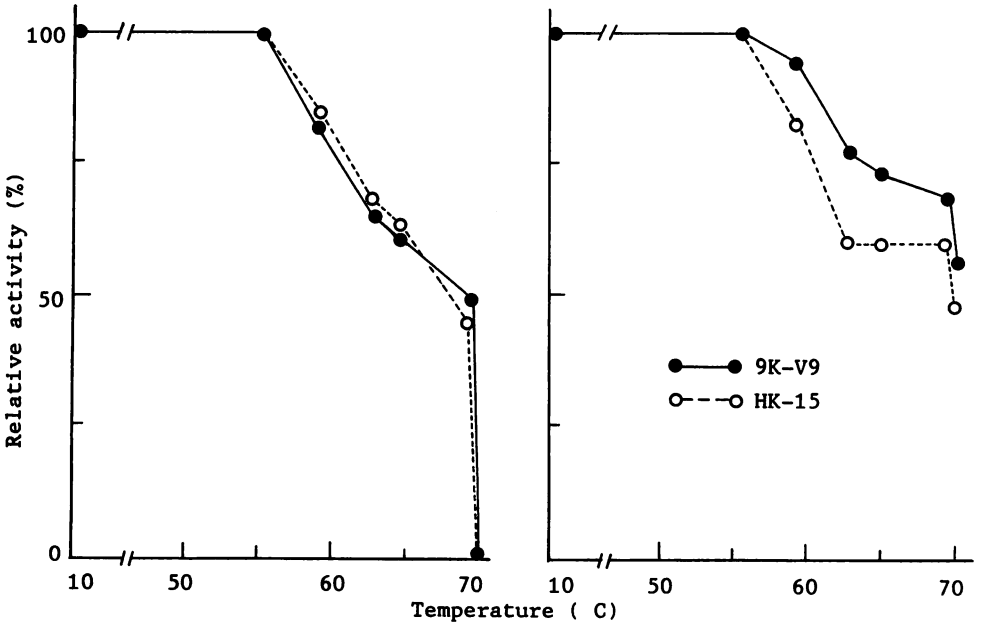

Fig. 7. Effect of heat treatment on the stability of antibiotic substance. Antibiotic activity was determined after incubation of Fraction A at different temperatures for 10 min in 0.05 M Tris-HCl buffer (left) or the same buffer containing 10 mM $MgCl_2$ (right).

Discussion

Marine purple bacteria belonging to the species *A. luteoviolacea*, have been reported by GAUTHIER[4] to produce macromolecular and low molecular antibiotics. The macromolecular antibiotic substance was characterized by polyanionic polysaccharide compound, autointoxication and antibiotic activity at the level of the respiratory chain. *A. luteoviolacea* strain 9K-V9 isolated from sea water of Kinko Bay, was also shown in the previous papers[1-3] to synthesize macromolecular antibiotic substance(s) which inhibit the growth of *A. luteoviolacea* strain itself as well as other test bacteria. This antibiotic substance was considered to be bound to the cells and released to the culture medium after the late logarithmic phase of bacterial growth.

The extracts from the cells contained lower molecular weight component (50,000) and higher molecular weight component responsible for antibiotic activity as shown on PAGE profiles. The latter component is considered to be the combined form of lower molecular one with other cellular materials.

Antibiotic substance from the strain 9K-V9 is shown to be heat-labile, precipitation with acetone or ammonium sulfate and staining with the dye for protein. These features indicate that the antibiotic substance from the strain 9K-V9 is composed of protein or proteinous compound containing other components.

In contrast to the results reported by GAUTHIER[5], the antibiotic substance produced by the strain 9K-V9 did not increase oxygen uptake by sensitive bacteria (data not shown). These results suggest that the strain 9K-V9 produces macromolecular antibiotic substance different from of *A. luteoviolacea* described by GAUTHIER in various points such as the chemical structure and the mode of action.

The antibiotic substance from strain 9K-9V inhibited the colony forming ability of almost all bacterial cells in sea water samples as described in previous paper.[2] However, the effect of the antibiotic substance on the cells of *V. parahaemolyticus* growing rapidly on agar medium at pH 7.6 was significantly weak as compared with that on the cells growing under acidic condition (pH 6.3). These results suggest that the antibiotic substance from the strain 9K-V9 acts against the cell envelope of bacterial cells effectively when they are growing much slowly under unsuitable conditions.

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