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Isolation and Characterization of Bacteriolytic Enzyme from a Marine Bacterium *Alteromonas* sp. No. 8-R

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Abstract

Bacteriolytic enzymes produced by *Alteromonas* sp. No. 8-R in nutrient broth were purified by ultrafiltration, ammonium sulfate precipitation, gel filtration, and cation-exchange chromatography. The bacteriolytic fractions after gel filtration contained six protein bands (A to F) on polyacrylamide gel electrophoresis (PAGE), and Rf values of each of the protein band A, B, C, and D on PAGE corresponded to that of bacteriolytic band a, b, c, and d, respectively, on PAGE containing cells of *Micrococcus luteus* reported previously (Takamoto et al., 1994). Finally, enzyme purified by cation-exchange chromatography contained a main protein band (B) and an indistinct protein band (E). The main protein band corresponded to bacteriolytic band b and was estimated to have a molecular weight of 74 kDa. Optimum pH and temperature for the activity of partially purified enzyme were 8.0 and 40°C, respectively. It was inactivated completely by heating at 70°C for 10 min.

Key Words: Marine bacteria, Genus *Alteromonas*, Bacteriolytic enzyme

Introduction

It is well-known that bacteria produce several kinds of bacteriolytic enzymes i.e. peptidoglycan hydrolase, containing N-acetylmuramidase, N-acetylglucosamidase, N-acetylmuramyl-L-alanine amidase, endopeptidase, and transglycosidase (Ghuysen et al., 1966). Peptidoglycan hydrolase is a range of endogenous enzymes and appears to be necessary for extension of cell wall and cell separation during bacterial growth, and also play some role in autolysis of cells. These knowledge of such enzymes have been obtained mostly from terrestrial bacteria. But, there is little information about similar enzymes produced by marine bacteria, and their enzymatic properties has not been determined yet.

A marine bacterium belonging to genus *Alteromonas* was the causative agent of red-spot injury on the seed twines of cultured makonbu *Laminaria japonica* (Ezura et al., 1988). The bacterium had at least four enzymes produced in different process during the growth in nutrient broth (Takamoto et al., 1994). The aim of the present study was to isolate, purify and partially characterize the bacteriolytic enzymes of *Alteromonas* sp. No. 8-R.

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Materials and methods

Bacterium and media

Alteromonas sp. No. 8-R isolated from red-spotted culture bed of makonbu, *Laminaria japonica* (Yumoto et al., 1989) was used in this study. The organism was grown at 20°C on slant of CSY-3 medium containing Difco casitone 1.0 g, Difco bacto-soytone 1.0 g, Difco yeast extract 1.0 g, ferric ammonium citrate (Wako) 0.1 g, agar (Wako) 13.0 g, and 1,000 ml natural sea water (pH 7.5) and was transferred monthly. The liquid medium CYT broth containing Difco casitone 1.0 g, Difco yeast extract 1.0 g, tris-hydroxymethyl-aminomethane (Tris) 0.5 g, and 1,000 ml artificial seawater (pH 7.5), was used for preincubation of the strain and experiments on bacteriolytic enzyme.

Assay of bacteriolytic activity

Bacteriolytic activity was measured as the decrease in turbidity of a substrate cell suspension. Freeze-dried whole cells of *Micrococcus luteus* (Seikagaku kogyo) used as a substrate were suspended in 50 mM Tris-HCl buffer (pH 7.5). The suspension was heated at 100°C for 10 min and diluted with the same buffer to give an absorbance of 1.2 at 620 nm. A reaction mixture consisted of 0.5 ml of a sample and 2.5 ml of heat-killed cells suspension and was incubated at 40°C for 20 min. The decrease in turbidity of the mixture was measured by a spectrophotometer (Hitachi, type 124) at 620 nm. One unit of bacteriolytic activity was defined as a decrease in absorbance of 0.001 per min at 620 nm.

Isolation of bacteriolytic enzyme

A loop of cells from a stock culture was inoculated to 5 ml of CYT broth and incubated at 25°C for 24 h with agitation. One ml each of preincubated culture was inoculated into four Erlenmeyer flasks each containing 2.5 l CYT broth. The flasks were incubated at 25°C on a rotary shaker. After 54 h of incubation, the culture fluids from four flasks were centrifuged at 16,000 × g for 20 min. The culture supernatant of about 8,050 ml was concentrated to about 300 ml by ultrafiltration with a Lab Conco Cassette System (cut-off Mw 10,000, Millipore). The concentrate was subjected to ammonium sulfate precipitation (0.9 saturation). The precipitate obtained by centrifugation at 16,000 × g for 30 min was suspended in 50 mM Tris-HCl buffer (pH 7.5) and dialyzed against 3.0 l of the same buffer for 9 h. The concentrated culture supernatant obtained was applied to a column (2.64 × 60 cm) of Toyopearl HW-50F (Tosoh) equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The active fractions obtained from the gel filtration were applied to a column (2.64 × 40 cm) of P-Cellulose (Cellulose Phosphate powder p-11, Whatman Biosystem Ltd). After washing of the column with the same buffer, the enzyme was eluted with the same buffer containing a linear gradient of KCl from zero to 0.5 M at a flow rate of 24 ml per h. The active fractions were combined and named as the partially purified bacteriolytic enzyme. The whole procedure was carried out at 1 to 4°C.

Electrophoresis

The active fraction after gel filtration and the partially purified bacteriolytic enzyme were analyzed by polyacrylamide gel electrophoresis (PAGE). PAGE was

performed by using single-dimension 1.0 mm thick slab gels in the buffer system of Laemmli (1970). The stacking gel contained 2.5% acrylamide, 0.63% bis-acrylamide, 0.125 M Tris (pH 6.8), and 0.07% *N, N, N', N'*-tetramethylethylenediamine (TEMED). The separating gel contained 7.0% acrylamide, 0.19% bis-acrylamide, 0.375 M Tris (pH 8.8), and 0.07% TEMED. The samples loading buffer contained 7% glycerol, 0.0625 M Tris, and 0.005% bromophenol blue (pH 6.8). Electrophoresis was carried out at 1 to 4°C with a current of 30 mA. After electrophoresis, the separating gel was stained with silver nitrate.

Detection of bacteriolytic band by PAGE containing M. luteus

The concentrated culture supernatants from 24, 36, 48 and 54 h incubations were analyzed by PAGE containing cells of *M. luteus* according to the modified procedure of Sugai et al. (1990) as mentioned previously (Takamoto et al., 1994).

Molecular weight determination

The molecular weight (M.W.) of the bacteriolytic enzymes after gel filtration was estimated by native-PAGE according to Ferguson plots (Bryan, 1977). Non-denatured protein molecular weight marker kit (Sigma) with a M.W. range from 14.2 kDa to 54.5 kDa was used.

Properties of the partially purified enzyme

The effect of temperature on the activity of the enzyme was measured by incubation for 20 min at the following temperatures: 20, 30, 40, 50 and 60°C. The effect of pH on the activity was measured by using the following buffers: 50 mM sodium acetate buffer (pH 4-6), 50 mM Tris-malate buffer (pH 6-7), 50 mM Tris-HCl buffer (pH 7-8), and 50 mM Glycine-NaOH buffer (pH 8-9.5). The assays were performed at 40°C for 20 min. Heat stability of the enzyme was determined as follows: after preincubation of the enzyme for 10 min at 10, 20, 30, 40, 50, 60, and 70°C, the activities were measured at 40°C for 20 min. Effect of salts and inhibitors on the enzyme activity were determined as follows: after preincubation of reaction mixtures containing CaCl₂ (1 mM, 0.1 M, and 0.01 M), MgCl₂ (1 mM, and 0.01 M), PCMB (1 mM), EDTA (1 mM), and DTT (1 mM), 50 mM Tris-HCl buffer (pH 7.5) at 1°C for 30 min, the activities were measured at 40°C for 20 min.

Results

Purification of the bacteriolytic enzyme

Purification of bacteriolytic enzyme from *Alteromonas* sp. No. 8-R was performed by four steps of ultrafiltration, ammonium sulfate precipitation, gel filtration, and cation-exchange chromatography. The results of the purification experiments are summarized in Table 1.

The culture supernatant from *Alteromonas* sp. No. 8-R was concentrated by using ultrafiltration followed by ammonium sulfate precipitation. The concentrated solution as fractionated by gel filtration on Toyopearl HW-50F was eluted in a single peak with bacteriolytic activity (Fig. 1). The active fractions (fraction no. 65-70) were applied to a cation-exchange column chromatography on P-Cellulose. Bacteriolytic enzyme was eluted at approximately 0.3 M KCl and the active peak

Table 1. Summary of partially purified bacteriolytic enzyme from the culture supernatant of *Alteromonas* sp. No. 8-R.

Step	Total volume (ml)	Total activity (units)	Specific activity (units/O.D. 280 nm)	Purification factor (-fold)	Yield (%)
Culture supernatant	8050	170660	14.7	1.0	100
Ultrafiltration	300	77100	72.6	5.0	45.2
Salting out	40	34080	76.7	5.2	20.0
Toyopearl HW-50F	454	9716	389	26.5	5.7
P-Cellulose	42	3520	2465	167.7	2.1

(fraction no. 80-90) coincided with the protein peak (Fig. 2). The final yield and specific activity of the bacteriolytic enzyme were 2.1% and 167.7 folds, respectively.

Comparison between bacteriolytic bands and protein bands

The bacteriolytic enzymes in culture supernatants prepared at 24, 36, 48, and 54 h of incubation were detected by PAGE containing the heat-killed cells of *M. luteus* (Fig. 3.A) which was similar as a previous report (Takamoto et al., 1994). The supernatants from 24 h and 36 h incubations contained only one lytic band (d). However the supernatant from 48 h incubation contained four lytic bands (a, b, c, and d), and lytic band d was not detected in the sample from 54 h of incubation.

The native PAGE of active fraction after gel filtration is shown in Fig. 3.B. Six protein bands (A, B, C, D, E, and F) were observed on the PAGE (lane 5). When Rf value of bacteriolytic bands and protein bands from PAGEs were compared, bacteriolytic band a, b, c, and d corresponded to protein band A, B, C and D, respectively. However, protein bands E and F (Fig. 3.B lane 5) did not correspond to any bacteriolytic bands. The enzyme obtained from final stage of purification contained 2 protein bands (B and E), and therefore, it can be considered that the enzyme was still in partially purified states.

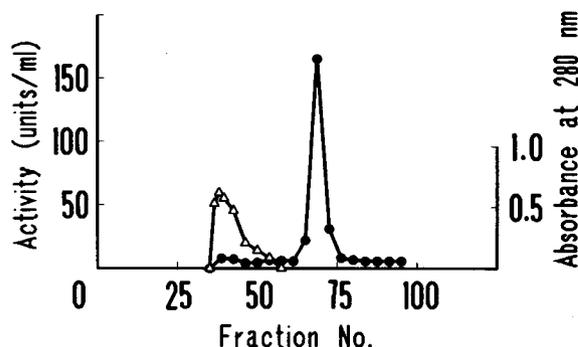


Fig. 1. Gel filtration of bacteriolytic enzyme from *Alteromonas* sp. No. 8-R on Toyopearl HW-50F.

Column size; 2.64 × 60 cm, Flow rate; 24 ml/h, Fraction size; 3 ml

●: Bacteriolytic activity, △: Absorbance at 280 nm

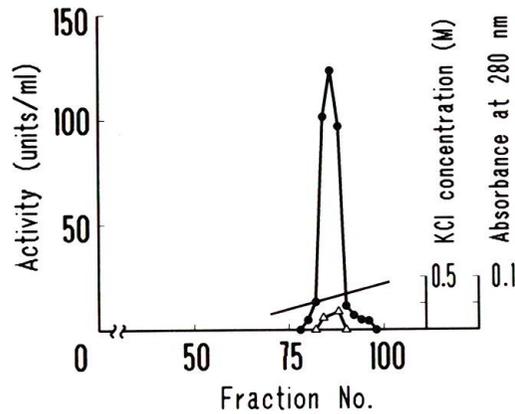


Fig. 2. Cation-exchange chromatography of the bacteriolytic enzyme from *Alteromonas* sp. No. 8-R on P-Cellulose.

Column size; 2.64 × 60 cm, Flow rate; 24 ml/h

●: Bacteriolytic activity, △: Absorbance at 280 nm, —: KCl concentration

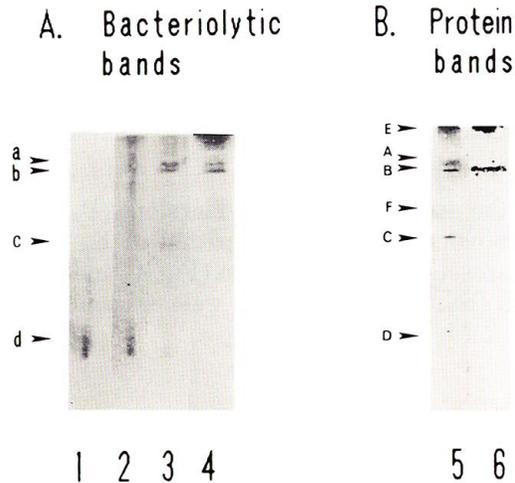


Fig. 3. Comparison between the bacteriolytic bands (A) and the protein bands (B) from *Alteromonas* sp. No. 8-R on native PAGE.

(A) The bacteriolytic enzyme profiles on PAGE containing cells of *Micrococcus luteus*. The samples were the culture supernatants at various incubation time.

Lane 1; 24 h, 2; 36 h, 3; 48 h, 4; 54 h, incubation.

(B) The protein bands on PAGE. The samples were active fractions from the gel filtration (lane 5) and the cation-exchange chromatography (lane 6).

Estimation of molecular weight

Molecular weights of protein bands B and C in the gel filtration were estimated by the method of Bryan (1977). Rf of the samples were determined on various concentrations of polyacrylamide gels: 5, 6, 7, 8, 9 and 10%, and 100 Log (Rf × 100)

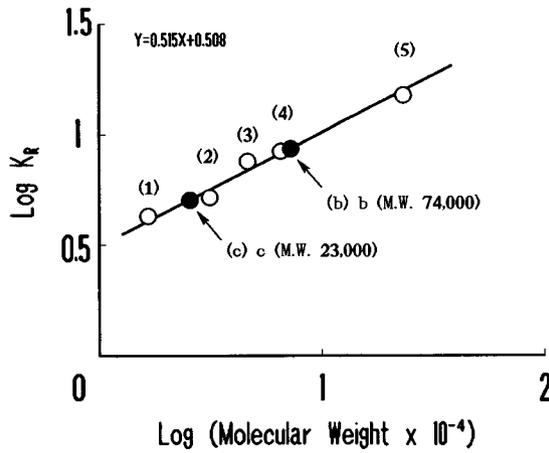


Fig. 4. Molecular weight estimation of the bacteriolytic enzymes by native-PAGE.

Open circles indicate standard proteins :

- (1) α -Lactalbumin of bovine milk (14.2 kDa)
- (2) Carbonic anhydrase of bovine erythrocytes (29 kDa)
- (3) Albumin of chicken egg (45 kDa)
- (4) Albumin monomer of bovine serum (66 kDa)
- (5) Urease trimer of jak bean (272 kDa)

Closed circles are the samples :

- (b) Protein band B (See Fig. 3)
- (c) Protein band C (See Fig. 3)

were plotted against the gel concentration. The slope (P_R : retardation coefficient) for each protein were calculated. The logarithm of K_R was plotted against the logarithm of molecular weight of each protein (Fig. 4) and M.W. of band B (bacteriolytic band b) and C (bacteriolytic band c) were estimated as 74 kDa and 23 kDa, respectively.

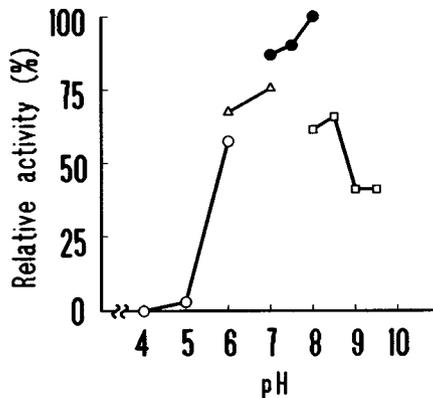


Fig. 5. Effect of pH on bacteriolytic activity of the partially purified enzyme from *Alteromonas* sp. No. 8-R.

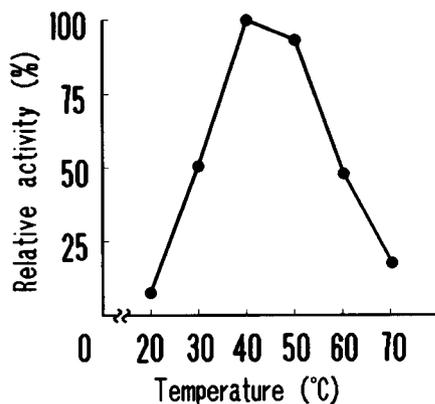


Fig. 6. Effect of temperature on bacteriolytic activity of the partially purified enzyme from *Alteromonas* sp. No. 8-R.

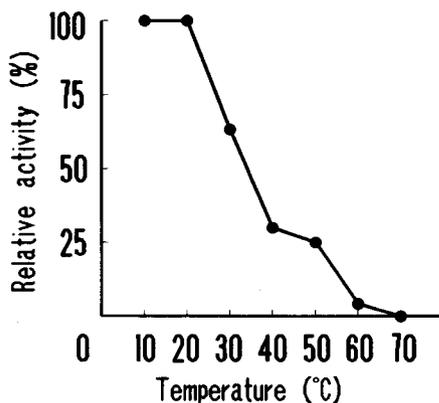


Fig. 7. Temperature stability of the partially purified enzyme from *Alteromonas* sp. No. 8-R.

Properties of the partially purified enzyme

The optimum pH for activity of the partially purified enzyme was about 8.0 (Fig. 5). The optimum temperature for the enzyme activity was about 40°C (Fig. 6). As shown in Fig. 7, the enzyme was stable at temperatures below 20°C. A half of initial activity was lost at 50°C for 10 min and it was inactivated completely by heating at 70°C for 10 min.

The effects of various chemical compounds and salts on bacteriolytic activities of the partially purified enzyme are shown in Table 2. The activities were not affected by the addition of DTT, but was slightly inhibited by EDTA, PCMB, 0.01-0.1 M CaCl₂ and 1 mM-0.1 M MgCl₂.

Table 2. Effect of CaCl₂, MgCl₂, EDTA, DTT and PCMB on bacteriolytic activity of the partially purified enzyme from *Alteromonas* sp. No. 8-R.

Chemicals	Relative activity (%)		
	0.1 M	0.01 M	1 mM
CaCl ₂	45	68	102
MgCl ₂	60	67	87
PCMB	—	—	66
EDTA	—	—	78
DTT	—	—	100

Substrate: heated cells of *Micrococcus luteus*

PCMB: p-chloromercuribenzoic acid

EDTA: ethylenediaminetetraacetic acid

DTT: dithiothreitol

Discussion

In this study, extracellular bacteriolytic enzymes of a marine bacterium *Alteromonas* sp. No. 8-R, were purified from the culture supernatant by ultrafiltration, ammonium sulfate precipitation, gel filtration and cation-exchange chromatography. Active fractions by gel filtration contained six protein bands A-F (Fig. 3B). In these protein bands, A, B, C, and D corresponded to bacteriolytic bands a, b, c and d respectively (Takamoto et al., 1994), according to these Rf values in PAGE, but protein bands E and F did not correspond to any bacteriolytic bands. After final purification of the enzyme by cation-exchange chromatography, it still contained two protein bands B and E (Fig. 3B) and was considered to be in partially purified state.

The band E migrated scarcely in PAGE. Some bacteriolytic enzymes have been known to be basic protein having the characteristics of high isoelectric point and no migration in PAGE without SDS treatment (Wadstrom, 1970, Yoshimoto et al., 1975, Sugai et al., 1990). But, in the preliminary investigation, no enzymatic activity was detected on SDS PAGE of the culture supernatant from the strain. And so, it may be assumed that the bacteriolytic activity of partially purified enzyme was caused by the enzyme b (corresponding to protein band B). The bacteriolytic enzyme b has a molecular weight of 74 kDa, an optimum pH of 8.0 and optimum temperature of 40°C for the activity, and was not stimulated by divalent cations.

It is well known that most of autolytic enzymes are cytoplasmic, a lower properties being associated with cell wall and are detected extracellularly at the death of cells (Croux et al., 1992). From the results of previous study (Takamoto et al., 1994), the bacteriolytic enzyme b is assumed to be an autolytic enzyme which was maintained intracellularly from the logarithmic phase to the stationary phase and appeared extracellularly at the later stages of the growth phase of the strain. The activity of the enzyme was not stimulated by divalent cations in contrast to what has been described for lytic enzymes of *C. botulinum* (Takumi et al., 1971) and

Bacillus subtilis (Rogers et al., 1984). And, many bacteriolytic enzymes have lower optimum pH (than 7.0) for their activity which are near to pH ranges of cell wall proteins (Croux et al., 1992).

From these evidences, it can be suggested that bacteriolytic enzymes of marine bacteria may have different properties from that of terrestrial bacteria. To confirm this speculation, further work is required to clarify the reaction mechanism and the other properties of the b and the other enzymes.

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