Biopolymers from Marine Invertebrates. XII.[1] A Novel Cytolytic Factor from a Hermit Crab, *Clibanarius longitarsus*

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Bioactive polymers were sought in marine arthropoda and a novel cytolytic factor was found in a hermit crab, *Clibanarius longitarsus*. The partially purified factor showed activity in fractions corresponding to a molecular weight of about 10 kilodaltons on a Sephadex G-75 column. This cytolytic factor was halfmaximally active for tumor cells at 0.13—0.66 μg/ml and for normal cells at 1.9—82 μg/ml. Tumor lysis by the factor was time dependent and was complete within 12 h. This bioactive polymer was labile on heating, at low and high pH.

Keywords cytolytic factor; hermit crab; marine animal; antitumor factor; *Clibanarius longitarsus*

Introduction

Marine animals have been reported to contain substances not found in terrestial animals,[2-4] which develop in a different environment. Moreover, marine invertebrates may contain special host defense factors, because their defense mechanisms differ from the immune systems of highly developed vertebrates.[5] With this in mind, we have searched for antibacterial and antitumor factors in marine mollusks.[6] Similar defense factors may be commonly distributed in marine invertebrates, and we therefore, sought a bioactive substance in arthropoda. Here, we describe a new cytolytic factor from a hermit crab, *Clibanarius longitarsus*.

Materials and Methods

Collection of Marine Invertebrates Specimens of *arthropoda* were collected in Iwate, Kanagawa and Shizuoka, Japan, in June and July of 1991. The animals were frozen at −20 °C until use.

Cytolytic Assay Target MM46 tumor cells were collected from the peritoneal cavity of C3H/He mice. Target cells (5×10^3 cells) were incubated with or without a test preparation in wells (7 mm diameter) of flat-bottomed microplates in 0.2 ml RPMI 1640 medium containing 5% fetal calf serum for 18 h at 37 °C under CO_2 in air. Cytolysis was defined by microscopic examination and MTT assay[7] as follows:

$$\% \text{ cytolysis} = 1 - \frac{\text{experimental viability}}{\text{control viability}} \times 100$$

Units of cytolytic activity were calculated as follows:

$$\text{units} = \frac{\text{final dilution giving 50\% cytolysis}}{1000}$$

Results

Cytolytic Activities of Various Arthropoda We initially checked which animals of marine arthropoda showed cytolytic activity. Each animal was homogenized with 2 volumes of 0.9% saline for 10 min, and the homogenate was centrifuged at 10000 rpm for 30 min. The supernatant was used for cytolytic activity assay. As shown in Table I, a hermit crab, *Clibanarius longitarsus* showed high cytolytic activity, and we therefore focused on the cytolytic factor of this animal.

We next examined which parts of the body showed the activity. The parts of the head and gill showed relatively high activity in the extract and parts of head and legs showed high total activity (data not shown). The activity of coelomic fluid was low. Since the cytolytic activity was not located in specific organs, however, we selected the whole body of a hermit crab as the starting material for purification of the cytolytic factor.

Partial Purification of Cytolytic Factor Specimens of the hermit crab, *C. longitarsus*, were collected in Lake Hamana, Shizuoka, Japan. The whole body without the shell was homogenized, and the supernatant of the homogenate (243 ml) of 50 bodies was dialyzed against 10 mM phosphate buffer. The dialyzed sample was loaded onto a column (2.9 × 42 cm) of DE52 (Whatman, Maidstone) previously equilibrated with the starting buffer (10 mM phosphate, pH 7.4). This column was washed with the starting buffer and then the adsorbed material was eluted with 1 M NaCl solution. The fractions with activity were combined, concentrated on an ultrafiltration membrane (Toyo Kagaku, Tokyo) and then applied to a column of Sephadex G75 (1.8 × 108 cm) (Fig. 1a). Fractions containing cytolytic factor were pooled, concentrated and applied to a second column (1.8 × 108 cm) of Sephadex G75 in phosphate buffered saline (Fig. 1b). The activity appeared in fractions corresponding to a molecular weight of about 10 kilodaltons (kDa). The active fraction also appeared in about 10 kDa on high performance liquid chromatography (LC-6A, Shimadzu) on a column (0.75 × 60 cm) of G3000 SW (Toyo Soda Manufacturing Co., Tokyo) (data not shown).

Table II summarizes the purification of the cytolytic factor. The specific activity was increased about 800 fold over the starting homogenate. The partially purified factor lysed target tumor cells over a concentration of 0.2 μg/ml (Fig. 2).

Characterization of the Cytolytic Factor The kinetics of cytolysis was studied (Fig. 3). Tumor lysis by the partial purified factor was time dependent and was complete within 12 h. After about 3 h, the number of bubbles in the

TABLE I. Cytolytic Activities of Various Arthropoda

Name	Species	Cytolysis (units/ml)
Barnacle	*Mitella mitella*	0.01
Crab	*Hemigrapsus sanguineus*	0.03
Acorn shell	*Tetraclita squamosa*	0.13
Sea louse	*Ligia exotica*	0.37
Hermit crab	*Pagurus samuelis* (Kanagawa)	0.06
	Pagurus samuelis (Iwate)	0.23
	Clibanarius longitarsus	2.25

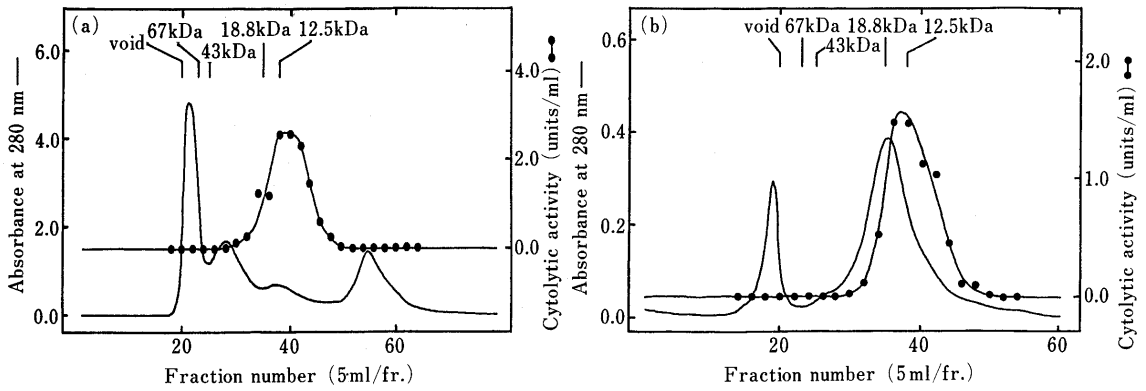

Fig. 1. Chromatography of the Cytolytic Factor on the First (a) and the Second (b) Sephadex G75 Column

The material was eluted with phosphate-buffered saline. Fractions (5 ml/fraction) were tested for cytolytic activity (●) and absorbance at 280 nm (—). Cytochrome c (12.5 kDa), myoglobin (18.8 kDa), ovalbumin (43 kDa) and bovine serum albumin (67 kDa) were used as marker proteins.

TABLE II. Purification of the Cytolytic Factor from a Hermit Crab

Step	Activity (units)	Protein[a] (mg)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Homogenate	57	3218	0.02	1	100
Dialysis	131	1944	0.07	4	231
DE52	39	41	0.95	53	68
Sephadex G75(I)	37	10	3.61	200	65
Sephadex G75(II)	62	4	15.5	827	110

a) Protein was measured by the procedure of Lowry *et al.*[9]

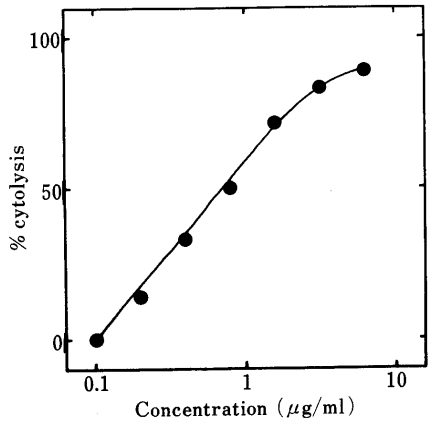

Fig. 2. Dose-Dependence of the Cytolytic Effect of the Factor

MM46 tumor cells were incubated with the indicated dose of the factor for 18 h.

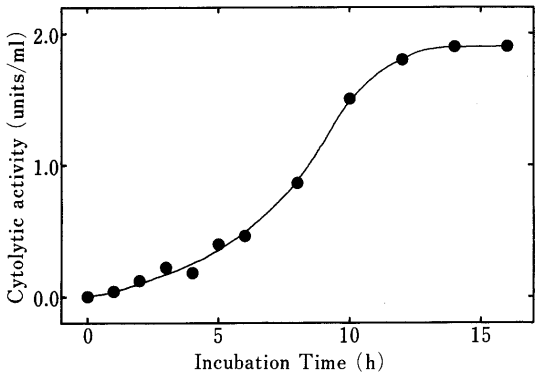

Fig. 3. Time Course of Cytolysis

MM46 tumor cells were incubated with the cytolytic factor at the times indicated.

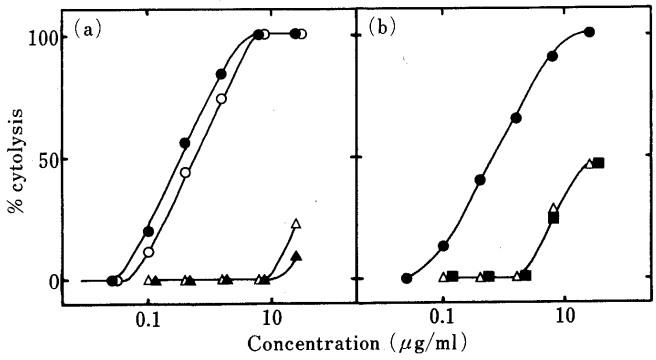

Fig. 4. Effects of the Heat Treatment (a) and pH Change on the Stability of the Cytolytic Factor (b)

The cytolytic factor was heat-treated for 10 min at the temperature indicated. ●, 37 °C; ○, 50 °C; △, 55 °C; ▲, 60 °C. The factor was treated for 30 min at the pH indicated. ●, pH 7; △, pH 4.5; ■, pH 9.

target cells gradually increased, and after 8 h the cell surface membrane burst.

Examination of the cytolytic activity against various target cells showed that the tumor cells tested were effectively lysed by this cytolytic factor (data not shown). In contrast, normal spleen cells and thymocytes were relatively resistant to the action of the factor. Tumor cells are thus more susceptible to this factor and the factor is not hemolysin.

Figure 4 shows the sensitivity of this cytolytic factor to pH and heat treatment. The factor was heat labile, showing an appreciable loss of activity after heating at 55 °C for 10 min (Fig. 4a). It was stable at neutral pH but lost its activity at pH 4.5 and pH 9 (Fig. 4b).

Discussion

Marine invertebrates may contain special host defense factors because their defense mechanisms differ from the immune systems of highly developed terrestial vertebrates.[5] We have already found 6 antibacterial and antitumor glycoproteins in the species *Aplysia* of a large shell-less opisthobranch of mollusk.[6] Here, we searched for a cytolytic factor in a marine arthropoda and found a new one in a hermit crab, *C. longitarsus*, a crustacea of arthropoda. Similar defense factors may be commonly distributed in marine invertebrates other than mollusk

and arthropoda. These cytolytic factors of invertebrates may correspond to cytolytic cytokines such as the tumor necrosis factor in vertebrates.

In crustacea, host defense factors such as lectin, hemolysin and antibacterial factors have reported,[8] but no cytolytic factor of a high molecular weight has yet been isolated from a hermit crab.

The physiological function of this cytolytic factor is not known at present. The activity was found in the gill, so that the factor may take part in cytolysis and/or act to eliminate small organisms in the gills of this species. The factor may be one component of a host defense system. Further studies are required to clear this point and for the further purification of this cytolytic factor.

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