Inhibitory effect of halocyamine, an antimicrobial substance from ascidian hemocytes, on the growth of fish viruses and marine bacteria

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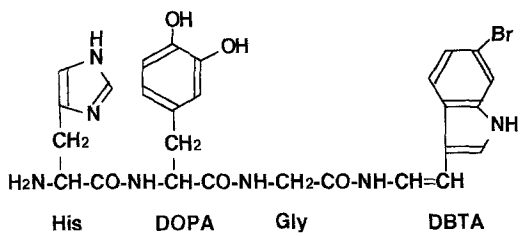
Summary. Halocyamine A, an antimicrobial substance isolated from hemocytes of the solitary ascidian *Halocynthia roretzi*, inhibited in vitro the growth of fish RNA viruses (infectious hematopoietic necrosis virus and infectious pancreatic necrosis virus). Pretreatment of RNA virus with halocyamine A reduced the infectivity of the virus toward host cells. The growth of marine bacteria, *Achromobacter aquamarinus* and *Pseudomonas perfectomarinus*, was also inhibited by halocyamine A but that of *Alteromonas putrefaciens* and *Vibrio anguillarum* was not. These results suggest that halocyamine may have a role in the defense mechanisms of *H. roretzi* against marine viruses and bacteria.
Key words. Halocyamine; antiviral substance; antibacterial substance; ascidian; hemocyte.

Several antiviral or antimicrobial substances, such as didemnins [1] and eudistomins [2, 3], have been isolated from ascidians. Didemnins isolated from the colonial ascidian *Tridemnum* sp. are cyclic peptides with a high level of antiviral and antitumor activity. They inhibit replication of various RNA and DNA viruses in vitro at low concentrations (0.05 ~ 1 μg/ml). Eudistomins isolated from the colonial ascidian *Eudistoma olivaceum* were found to be highly active agents against herpes simplex virus. The eudistomins are β-carboline derivatives containing bromine atoms. Almost all of them have been isolated from whole animal extracts. Therefore, we do not know in which tissue they exist, and we cannot make any assumptions about how they function in the immune systems of ascidians.

It has been proposed that hemocytes play important roles in the defense mechanisms of ascidians [4]. We found two antibacterial substances, halocyamine A and B (fig. 1), in hemocytes of the solitary ascidian *Halocynthia roretzi* [5]. They were tetrapeptide-like substances containing one bromine atom and were present only in the hemocytes. Both possess antimicrobial activity against several kinds of gram-positive bacteria and yeasts, and against a highly antibiotic-sensitive strain of a gram-negative bacterium. They also show cytotoxic activity against some cultured mammalian cells. We examined the effect of halocyamine A on the growth of fish disease viruses and marine bacteria.

Halocyamine A



Halocyamine B

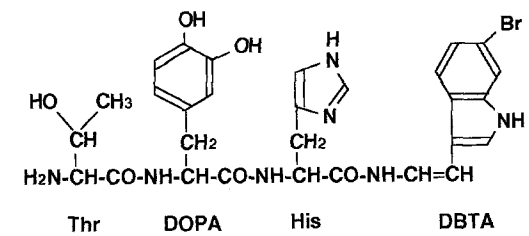


Figure 1. Structures of halocyamine A and B.

Materials and methods

Halocyamine A was isolated as described previously [5] and kept at − 20 °C until used.

An established cell line, RTG-2, derived from trout ovary [6], was cultured in modified Eagle's medium (MEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 IU/ml penicillin G (Sigma), and 100 μg/ml streptomycin sulfate (Sigma) (10% FBS-MEM). The cells were infected by two fish viruses, infectious hematopoietic necrosis virus (IHNV), a Rhabdovirus, and infectious pancreatic necrosis virus (IPNV), a Birnavirus. Virus particles released from the cells were collected and stored at − 80 °C until used. Antiviral activity of halocyamine A was assayed by measuring the effect on plaque formation [7]: RTG-2 cell (10^6 cells/ml) in a volume of 1 ml of 10% FBS-MEM were added to each well of a 16-mm multiple well plate and cultured at 15 °C for 24 h. After removing the medium, 0.1 ml of virus solution was added and the virus was allowed to be adsorbed to the cells for 1 h. After the well had been washed twice with Hank's balanced salt solution (Gibco), 0.1 ml of halocyamine A, dissolved in dimethylsulfoxide and diluted to give the appropriate concentration, was added and then 0.9 ml of 2% FBS-MEM containing 0.8% methylcellulose was layered over the cells. The cells were cultured at 15 °C for 7 days. The number of plaques formed was counted after fixation with 10% formalin

and staining with 0.1 % crystal violet (experiment 1). Duplicate measurements were carried out at each concentration of halocyamine A and then the mean value was calculated.

To investigate which step of the life cycle of the virus is affected by halocyamine A, two additional experiments were carried out. First, IHNV (0.1 ml) and halocyamine A (0.1 ml) were mixed to give a final halocyamine concentration of 50 µg/ml and incubated at 15 °C for 3 h, and then the mixture was added to the cultured cells and allowed to stand for 1 h. After washing, 2% FBS-MEM containing 0.8% methylcellulose was layered over the cells. The cells were treated as described above (experiment 2). Secondly, halocyamine A was added to the cultured cells to give a concentration of 50 µg/ml and the cells were cultured for 24 h. After washing, the cells were infected by IHNV. The infected cells were then incubated for 1 h and treated as described above (experiment 3). Experiments 2 and 3 were also carried out in duplicate and the mean value was calculated in each experiment.

Antibacterial activity of halocyamine A against marine bacteria was measured as follows. Four marine bacteria (*Achromobacter aquamarinus* ATCC 14400, *Alteromonas putrefaciens* IAM 12079, *Pseudomonas perfectomarinus* ATCC 14405, and *Vibrio anguillarum* NCMB 828) were precultured at 25 °C for 2 days in ZoBell's media[8]. A suspension of each bacterium was prepared, and the absorbance at 660 nm adjusted to 0.8. To 25 µl of each bacterial suspension were added 950 µl of ZoBell's medium. Twofold serial dilutions of halocyamine A (initially dissolved in 80% methanol) were prepared, and 25 µl added per tube. The bacteria were allowed to grow at 25 °C for 35 h. Then, the absorbance at 660 nm of each bacterial suspension was measured. The mean value was calculated from the data of duplicate measurements.

Results and discussion

Halocyamine A showed an inhibitory effect in vitro on the growth of fish RNA viruses (IHNV and IPNV) in RTG-2 cells (fig. 2). It completely inhibited the growth of both viruses at the concentration of 100 µg/ml, at which the host cells did not undergo lysis. So the results indicate that halocyamine A has an inhibitory effect on viruses but not a cytotoxic effect on host cells. The concentrations required for 50% inhibition of the growth of IHNV and IPNV were estimated to be about 40 and 60 µg/ml, respectively. It should be noted that halocyamine A showed antiviral activity against IPNV; antiviral reagents against this virus have been sought before, but not found. When the host cells were first treated with halocyamine A and then infected by IHNV after washing, halocyamine A showed little antiviral activity (fig. 3, experiment 3). On the other hand, when IHNV was first treated with halocyamine A and then added to the host cells, the compound showed antiviral activity (fig. 3, experiment 2) as strongly as was the case when halocyamine A was added after adsorption of the virus to the host cells had been completed (fig. 3, experiment 1). These results suggest that halocyamine A can act on the viruses themselves but not on the host cells, and that it causes some damage to the viruses which prevents them from attacking the host cells. Further studies are necessary to define the precise action of halocyamine A on viruses.

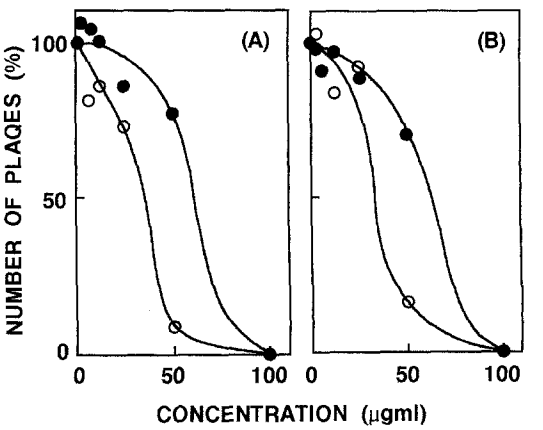


Figure 2. Antiviral activity of halocyamine A against the growth of IHNV (o) and IPNV (●). The results obtained from two repeated experiments are illustrated in (*A*) and (*B*). Each point represents the mean value calculated from the data of duplicate measurements. Number of plaques in the absence of halocyamine A was defined as 100%.

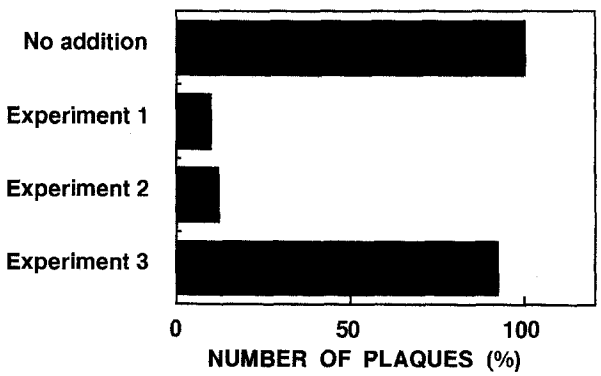


Figure 3. Effects of pretreatment with halocyamine A. In experiment 1, the effect of halocyamine A was measured after IHNV was adsorbed to the host cells. In experiment 2, IHNV was first treated with halocyamine A. In experiment 3, the host cells were first treated with halocyamine A. The final concentration of halocyamine A used in each experiment was 50 µg/ml. No addition represents number of plaques in the absence of halocyamine A, which was defined as 100%.

Halocyamines are more effective against gram-positive bacteria such as *Bacillus subtilis* than against gram-negative ones such as *Escherichia coli*[5]. Most marine bacteria, including *A. aquamarinus*, *A. putrefaciens*, *P. perfectomarinus*, and *V. anguillarum*, all of which were used in this study, are gram-negative. Halocyamine A also showed an inhibitory effect on the growth of some marine bacteria such as *A. aquamarinus* and *P. perfectomarinus* (the concentrations required for 50% inhibition were 60 and 75 µg/ml, respectively), but little effect on that of *A. putrefaciens* and *V. anguillarum* (fig. 4). Halo-

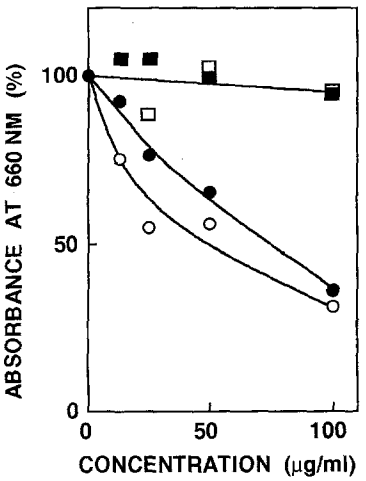


Figure 4. Antibacterial activity of halocyamine A against marine bacteria. The absorbance at 660 nm in the absence of halocyamine A in each bacterium was defined as 100%. *A. aquamarinus* (○), *P. perfectomarinus* (●), *A. putrefaciens* (□), and *V. anguillarum* (■).

cyamine B showed antibacterial spectra similar to those of A (not shown). The reasons for lack of antimicrobial activity of halocyamines against the latter two bacteria remain unknown.

Antimicrobial substances[9] and lectins[10,11] have been proposed as humoral factors in the defense mechanisms of invertebrates. In ascidians, lectins have been proposed as recognition molecules for foreign substances[12]. With respect to antibacterial substances in ascidians, we have already proposed that halocyamines present in hemocytes may have an extracellular function in the defense mechanisms of *H. roretzi*[5]. It has been demonstrated that *H. roretzi* hemocytes undergo several cellular responses such as phagocytosis[13] and lysis (contact reaction[14]) against foreign (non-self) substances.

The results described in this paper showing that halocyamines have an inhibitory effect on the growth of two kinds of fish disease viruses and two kinds of marine bacteria, support the above assumption that halocyamines could function as defense agents against viruses and/or bacteria invading the hemolymph of *H. roretzi*. The antiviral and antimicrobial activities of the halocyamines described in this study were lower than those of antiviral and antimicrobial substances isolated from colonial ascidians[1-3] by approximately two orders of magnitude. In this connection, we have shown that the combined concentrations of the two halocyamines accumulated in only one type of hemocyte, a vesicular cell[13], can reach a level as high as 10 mg/ml[5]. This type of cell was the most abundant (more than 50%) hemocyte in the hemolymph of *H. roretzi*. Therefore, the total concentrations of halocyamines could well be high enough to attack viruses and bacteria invading the hemolymph.

In preliminary studies, we found that halocyamine A showed acute toxicity against mice at a concentration of more than 100 mg/kg. This fact raises a hope that halocyamine and its derivatives might be candidates for use as medicines applicable to some human diseases.

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Mitochondrial DNA variation in social wasps (Hymenoptera, Vespidae)

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Summary. Patterns of restriction fragment length polymorphisms (RFLP) of European Vespinae were more similar within genera than between them. Distance trees were constructed that support the hypothesis of monophyly of the genera *Vespula* and *Dolichovespula*. Within the genus *Vespula*, *V. germanica* was more closely related to *V. rufa* than to *V. vulgaris*. The position of the genus *Vespa* remained uncertain due to the precision limits of the RFLP technique.
Key words. Vespinae; mitochondrial DNA; restriction fragment length polymorphism (RFLP).