Biopolymers from Marine Invertebrates. X.[1] Mode of Action of an Antibacterial Glycoprotein, Aplysianin E, from Eggs of a Sea Hare, *Aplysia kurodai*

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An antibacterial factor, aplysianin E, was purified from the eggs of a sea hare, *Aplysia kurodai*. Purified aplysianin E was a glycoprotein of 250 kilo daltons consisting of 3 subunits, and showed both antibacterial and antineoplastic activities. The two activities were lost in parallel on heating and at low and high pH. This factor was half-maximally active for gram-positive and -negative bacteria at 0.12—3.3 μg/ml and its action was not bactericidal but bacteriostatic. Aplysianin E did not induce morphological elongation of bacteria or their release of adenosine triphosphate (ATP), but it completely inhibited the syntheses of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) by *E. coli* within 10 min. These results suggest that aplysianin E, found in an invertebrate, the sea hare, is a new antibacterial protein and that it exerts its action by inhibiting nucleic acid synthesis, as a DNA-inhibiting chemotherapeutic drug does.

Keywords antibacterial protein; biopolymer; marine animal; aplysianin E; sea hare; *Aplysia kurodai*

Marine animals, which develop in a different environment from terrestial animals, have been reported to contain substances not found in terrestial animals.[2-5] Invertebrates may contain special host-defense factors, because their defense mechanisms differ from the immune system of highly developed vertebrates.[6] In studies with this idea in mind, we have found several bioactive factors in marine animals.[7-11] Recently, we have reported novel antibacterial and antitumor factors (aplysianin) in sea hares of the *Aplysiomorpha*.[12-14] Here, we report the mode of action of aplysianin E on bacterial growth. We found that aplysianin E inhibits the growth of a variety of bacteria and their syntheses of macromolecules, suggesting that the primary target of this glycoprotein in bacteria is nucleic acid synthesis.

Materials and Methods

Collection of Eggs of *Aplysia* Species Eggs of *A. kurodai* were collected in Lake Hamana, Shizuoka, Japan, in the spawning season (May and June) and were stored at −20 °C until use.

Extraction of Aplysianin E Egg masses of *A. kurodai* were homogenized with 2 volumes of 0.9% saline for 10 min, and the homogenate was centrifuged at 10000 rpm for 30 min to obtain a clear supernatant, which was used as starting material for purification of aplysianin E.

Assay of Antibacterial Activity The medium used for growth of bacteria was antibiotic medium (Bacto Penassay Broth, Difco). Bacteria in the exponential phase of growth were collected and suspended in 10 mM phosphate buffer containing 130 mM NaCl at an absorbance (550 nm) of 0.1. The sample (100 μl) diluted with medium and the bacterial suspension (100 μl) were mixed in a flat-bottomed 96-well multiplate and incubated at 37 °C for 4—18 h with shaking. Then the mixture was rapidly chilled and its A_{550} was measured. For quantification of antibacterial activity, one unit of antibacterial activity was defined as the amount that caused 50% inhibition of bacterial growth relative to the control.

Assay of Lysis of Nucleated Cells The cytolytic activity of aplysianin E was determined as reported previously.[14] Briefly, ^{51}Cr-labeled MM46 tumor cells were incubated with or without a test preparation in wells containing 0.2 ml of RPMI 1640-fetal calf serum (10%) for 18 h at 37 °C under 5% CO_2 in air. The radioactivity of the supernatant was measured and units of cytolytic activity were calculated as follows:

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

Assay of Macromolecular Synthesis The metabolic activities of bacteria with and without treatment with aplysianin E were measured in terms of incorporation of tritiated thymidine, uridine, and leucine into deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein, respectively. Samples of bacteria were incubated with 1 μCi of [methyl-^{3}H]thymidine (52 Ci/mmol), 2 μCi of [5,6-^{3}H]uridine (39 Ci/mmol), or 5 μCi of L-[4,5-^{3}H]leucine (170 Ci/mmol) (all from New England Nuclear, Boston, MA) in 100 μl of phosphate-buffered saline at 37 °C for 4 h. Then the macromolecules were precipitated on filters with 5% trichloroacetic acid and washed with a Labo Mash LM-101 machine. The filters were dried and their radioactivity was counted in a liquid scintillation spectrophotometer.

Results

Purification of Aplysianin E Previously we found an antibacterial and antineoplastic glycoprotein in the eggs of *Aplysia kurodai*[11,13] and purified it as aplysianin E.[14] The antibacterial and antineoplastic activities were not separated by column chromatography (Fig. 1); purified aplysianin E shows both activities.

Here for the first time we have purified aplysianin E in large quantity by ion exchange chromatography and two types of gel filtration as reported previously.[14] Table I summarizes the purification of aplysianin E from the eggs of *A. kurodai*. About 39 mg of pure protein was obtained from 380 g of eggs. The specific activity of the purified material was increased about 29-fold over that of the crude homogenate, and on electrophoresis the preparation gave three main bands of 76, 88 and 102 kilo daltons (kDa) (data not shown). These data suggest that the purity of aplysianin E was nearly the same as that of the purified material obtained previously.[14]

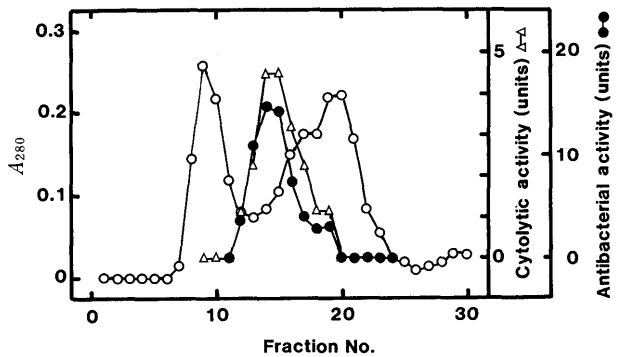

Fig. 1. Elution Patterns of Applysianin E on Column Chromatography

A homogenate of *Aplysia* eggs was applied to a Sepharose 6B column (1 × 30 cm). Fractions (1 ml) were tested for cytolytic activity (△), antibacterial activity (●) and absorbance at 280 nm (○)

This aplysianin E inhibited the growth of *E. coli* at a concentration of 0.4 μg protein/ml and that of *S. aureus* at 0.13 μg protein/ml (Fig. 2). As shown in Table II, aplysianin E inhibited the growth of all the bacteria tested, including both gram-positive and -negative strains. Therefore, aplysianin E seems to be an antibacterial factor with a wider spectrum of activity than usual antibiotics or chemotherapeutic drugs.

Characterization of Aplysianin E We first examined the stability of the antibacterial activity of aplysianin E. As shown in Fig. 3A, the factor was stable at neutral pH, but lost half its activity at pH 12 and all its activity at pH 2. Aplysianin E was heat-labile, showing appreciable loss of activity after heat-treatment at 60 °C for 10 min (Fig. 3B). These treatments caused loss of the antibacterial and antineoplastic activities simultaneously (Fig. 3A and 3B), suggesting that the active sites for the two activities are similar or identical.

To determine the antibacterial mechanism of aplysianin E, we examined whether the factor showed bactericidal or bacteriostatic activity. As shown in Fig. 4, the growth of *E. coli* stopped immediately after the addition of aplysianin E at 10 μg/ml, but the factor did not lyse the bacteria, in contrast to the bactericidal drug ampicillin.

Next, we examined the effect of aplysianin E on the adenosine triphosphate (ATP) pool of bacteria. As shown in Fig. 5, like ampicillin, aplysianin E did not affect the ATP pool of *E. coli*. Moreover, aplysianin E did not induce the release of ATP from *E. coli* (data not shown).

We also examined the correlation between cell metabolism and antibacterial activity. Figure 6 shows that the abilities of bacteria to incorporate thymidine and uridine were completely inhibited within 4 h after addition of the factor, as well as by nalidixic acid, a DNA-inhibiting chemotherapeutic drug. Thus, the growth inhibition by aplysianin E may be due to decreased metabolic activities

TABLE I. Purification of Aplysianin E

	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (unit/mg)	Purification (fold)	Yield (%)
Crude	1130	18080	5368	3	1	100
Dialyzed	1168	18693	2921	6	2	103
DE-52	26	11778	592	20	6	65
Sepharose 6B	7.5	6795	99	68	20	38
Sephacryl S-300	10.6	3837	39	98	29	21

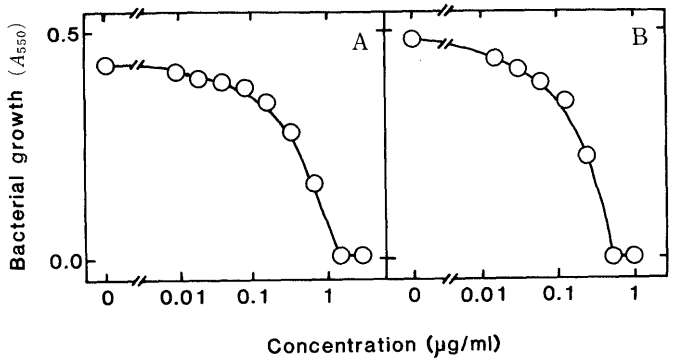

Fig. 2. Dose-Dependence of Antibacterial Activity of Aplysianin E

E. coli (LE-392) (A) and *S. aureus* (B) were incubated with or without aplysianin E for 4 h.

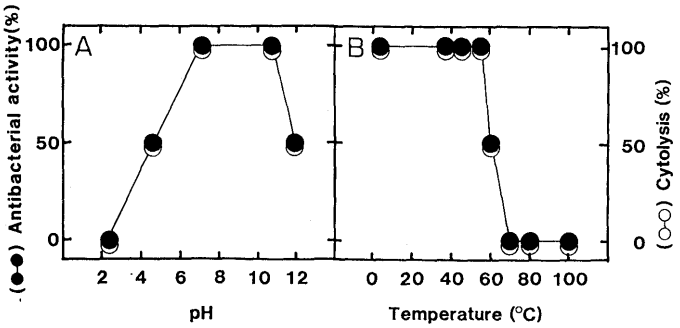

Fig. 3. Stability of Aplysianin E at Various pHs and Temperatures

Aplysianin E (10 μg/ml) was incubated at pH 2—12 for 30 min (A) or at 0—100 °C for 10 min (B). Its antibacterial (●) and cytolytic (○) activities after the treatment are expressed as residual activities (percentages of those of the untreated control).

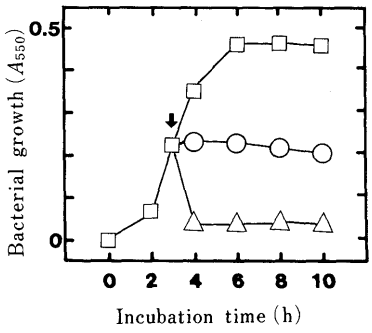

Fig. 4. Time Course of Antibacterial Effect of Aplysianin E

E. coli (LE392) was incubated with aplysianin E (○) or ampicillin (△), or without either (□). ↓; Addition of aplysianin E (10 μg/ml) or ampicillin (10 μg/ml).

TABLE II. Target Specificity of Antibacterial Factors

Target cell[a]	IC_{50} (μg/ml)					
	Aplysianin E	Ampicillin	Cefalotin	Cefalexin	Nalidixic acid	Pipemidic acid
Escherichia coli (LE-392)	0.40	0.39	1.2	1.5	6.6	1.6
Enterobacter cloacae	0.68					
Klebsiella pneumoniae	0.18					
Serratia marcescens	0.30					
Salmonella typhimurium	3.30					
Aeromonas hydrophila	2.5	1.3	>20	16	0.32	0.48
Staphylococcus aureus	0.13	0.004	0.023	0.033	>2	>2
Staphylococcus epidermidis	0.12					
Streptococcus sp. (SG8004)	0.44	0.001	0.005	0.005	>2	>2

a) The sample and bacteria suspension were incubated at 37 °C for 18 h except *E. coli* (4 h).

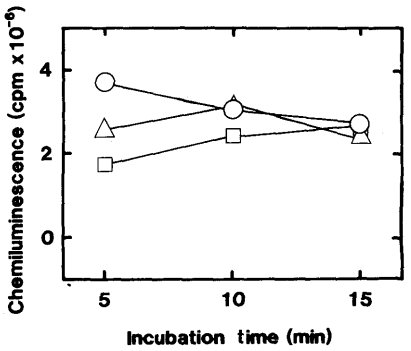

Fig. 5. Effect of Aplysianin E on the ATP Pool of *E. coli*

Aplysianin E (10 μg/ml) (○) or ampicillin (10 μg/ml) (△) was added to a suspension of *E. coli* and samples were taken at the indicated times. ATP was measured by luciferin/luciferase assay in a Lumicounter 1000 (Nichion). □, control.

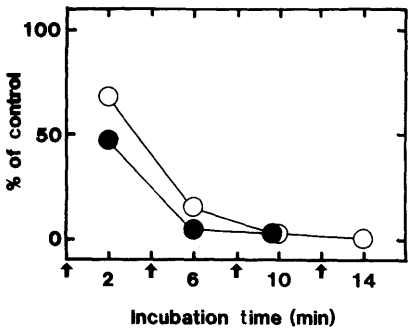

Fig. 7. Effects of Aplysianin E on Pulse Labelling of Nucleic Acids

E. coli was incubated with or without aplysianin E (10 μg/ml). Ten μCi/ml of tritiated thymidine (○) or uridine (●) was added at the points indicated by arrows and the mixtures were incubated at 37 °C for 2 min. The acid-insoluble fraction was obtained and its radioactivity was measured.

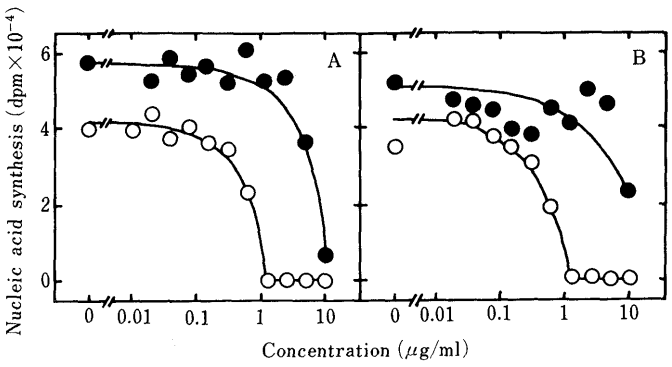

Fig. 6. Effects of Aplysianin E on Synthesis of Nucleic Acids in *E. coli*

E. coli was incubated with or without aplysianin E (○) or nalidixic acid (●), and then with 10 μCi/ml of tritiated thymidine (A) or uridine (B) at 37 °C for 4 h. The acid-insoluble fraction was obtained and its radioactivity was measured.

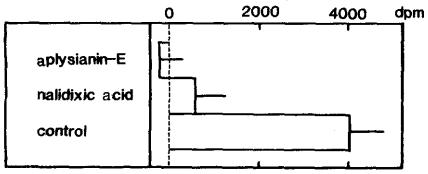

Fig. 8. Effects of Aplysianin E on DNA Synthesis in *E. coli*

Tritiated thymidine (100 μCi/ml) was added to *E. coli* at 37 °C for 5 min and then the cells were washed. Aplysianin E (10 μg/ml) or nalidixic acid (100 μg/ml) was added to these cells with incorporated thymidine and incubated at 37 °C for 20 min. The acid-insoluble fraction was then obtained and its radioactivity was measured.

such as the synthesis of DNA and RNA. The results of a pulse experiment in Fig. 7 show that aplysianin E inhibited the synthesis of nucleic acids in bacteria completely within 10 min. Moreover, like nalidixic acid, aplysianin E inhibited DNA synthesis in cells containing ^{3}H-thymidine (Fig. 8) and did not inhibit the incorporation of ^{3}H-thymidine into cells (data not shown), suggesting that it did not affect the transport systems of bacteria.

Discussion

Sea hares of the species *Aplysia* belong to the subclass opisthobranchia of the mollusca. They have been reported to contain various biologically active substances, including antibacterial factors,[15] cytolytic factors[16,17] toxins[18] and chemical defensive substances.[19,20] Most of these substances are low-molecular-weight compounds derived from the algae on which the sea hares feed. However, no antibacterial proteins except aplysianins[13,21] have been found in sea hares.

The species *Aplysia* lay yellow eggs in gelatinous strings in the spawning season (May and June). Although these eggs appear defenseless, they do not seem to be invaded by bacteria or eaten by predators. These observations suggest that the eggs contain some biologically active substance for their protection. In fact, we found an antibacterial glycoprotein, aplysianin E, in the eggs.[21] It is not surprising that marine mollusca such as sea hares have antibacterial proteins, because terrestial animals such as mammals, amphibians and insects contain a variety of antibacterial proteins.[22-24]

Previously we reported that aplysianin E was a 250-kDa glycoprotein consisting of three different subunits. This factor was half-maximally active at 2—114 ng protein/ml and lysed all the tumor cells tested, but did not lyse normal white or red blood cells.[14] Here, we purified aplysianin E in large quantity and examined its antibacterial action. Aplysianin E inhibited the growth of all the bacterial strains tested at 0.13—3.3 μg protein/ml. Its action was not bactericidal, but bacteriostatic, and so bacteria grew after removal of aplysianin E. Aplysianin E did not induce morphological elongation of bacteria (data not shown), suggesting that it did not inhibit cell wall synthesis. Moreover, it did not cause release of ATP from bacteria. It did inhibit the synthesis of DNA and RNA within a few minutes. Therefore, the antibacterial action of aplysianin E may be due, not to inhibition of cell wall synthesis or energy metabolism, but to inhibition of nucleic acid synthesis, like that of a DNA-inhibiting chemotherapeutic drug. The mechanism of its effect in inhibiting nucleic acid synthesis of bacteria requires study.

The antibacterial protein, aplysianin E, also shows antineoplastic activity.[14] Most antitumor antibiotics also show both antibacterial and antineoplastic activities. Since aplysianin E inhibited the synthesis of nucleic acids in tumor cells,[14] these two activities may share a common mechanism in terms of inhibition of nucleic acids synthesis. Moreover, these two activities of aplysianin E were lost in parallel during various treatments, suggesting that the active sites for the two activities may be similar or identical.

In the present work we found that a marine mollusc, like terrestial animals, contains an antibacterial glycoprotein,

and that this protein inhibited the syntheses of nucleic acids in bacteria completely within a short time. The wide distribution of antibacterial proteins in the animal kingdom indicates that these proteins have been well conserved during evolution, which is understandable because animals cannot survive unless they can eliminate invading bacteria.

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