Biopolymers from Marine Invertebrates. XIII.[1] Characterization of an Antibacterial Protein, Dolabellanin A, from the Albumen Gland of the Sea Hare, *Dolabella auricularia*

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An antibacterial factor, dolabellanin A, was purified from the albumen gland of a sea hare, *Dolabella auricularia*. Purified dolabellanin A was a glycoprotein of 250 kilodaltons consisting of 4 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 0.018—0.48 μg/ml, and its action was not bactericidal but bacteriostatic. Dolabellanin A did not induce morphological elongation of bacteria or the release of adenosine triphosphate, but it completely inhibited the syntheses of deoxyribonucleic acid (DNA) and ribonucleic acid by *E. coli* within 6 min. These results suggest that dolabellanin A, which is found in a marine invertebrate, the sea hare, is a new antibacterial protein, and that it exerts its action by inhibiting nucleic acid synthesis, as does a DNA-inhibiting chemotherapeutic drug.

Keywords antibacterial protein; biopolymer; marine animal; dolabellanin A; sea hare; *Dolabella auricularia*

Introduction

The defense mechanisms of invertebrates differ from the immune system of highly developed vertebrates.[2,3] Thus, invertebrates may have host-defense factors other than those vertebrates have. Moreover, marine animals contain various substances not found in terrestrial animals,[4,5] because of the different environment in which they develop. Most of these substances, however, are low molecular weight compounds, and the high molecular compounds have not been well studied. We thus focused on biopolymers of marine invertebrates and found several bioactive factors in these animals.[6] We recently reported a novel antitumor glycoprotein, dolabellanin A, from the albumen gland of a sea hare, *Dolabella auricularia*.[7] Here, we report that dolabellanin A 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

Dolabella auricularia Specimens of *D. auricularia* were collected in Tateyama, Kominato, Aburatsubo and Kochi, Japan, in May, June and July. The animals were frozen at −20 °C until use.

Assay of Antibacterial Activity The medium used for growth of bacteria was an antibiotic medium (Bacto Penassay Broth, Difco). Bacteria in the exponential phase of growth were collected and suspended in 10 mM of a 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) was 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 dolabellanin A was determined as reported previously.[7] 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 (5%) 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:

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

Cytolytic assay was also microscopically performed.

Assay of Macromolecular Synthesis The metabolic activities of bacteria with and without treatment with dolabellanin A 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) or 2 μCi of [5,6-^{3}H]uridine (39 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 Dolabellanin A We purified dolabellanin A in large quantity by ion exchange chromatography and gel filtrations. Table I summarizes the purification of antibacterial glycoprotein from the albumen gland of *D.*

TABLE I. Purification of Dolabellanin A from Albumen Gland of *D. auricularia*

Step	Activity (units)	Protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Homogenate	171	471	0.36	1	100
DEAE-cellulose (I)	181	240	0.76	2.1	106
Sepharose 6B	129	49	2.66	7.3	75
Sephacryl S-300	102	61	1.66	4.6	59
DEAE-cellulose (II)	53	16	3.30	9.1	31

TABLE II. Target Specificity of Dolabellanin A

Target cell	50% inhibitory dose (ng/ml)
Escherichia coli (LE-392)	90
Escherichia coli (DH-1)	25
Escherichia coli (RR-1)	40
Escherichia coli (C-600)	85
Escherichia coli (JM 103)	70
Escherichia coli (JM 105)	46
Enterobacter cloacae	98
Klebsiella pneumoniae	21
Serratia marcescens	48
Salmonella typhimurium	480
Aeromonas hydrophila	260
Staphylococcus aureus	25
Staphylococcus epidermidis	18
Streptococcus sp. (SG8004)	63

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Table III. Antibacterial Activities of Dolabellanin A and Chemotherapeutic Drugs

	50% inhibitory dose (μg/ml)					
	Dolabellanin A	Ampicillin	Cefalexin	Cefalotin	Nalidixic acid	Pipemidic acid
E. coli	0.08	0.39	1.5	1.2	6.6	1.6
S. aureus	0.02	0.004	0.03	0.02	>2	>2

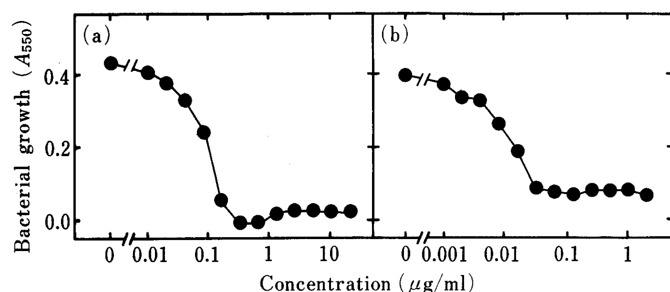

Fig. 1. Dose–Dependence of Antibacterial Activity of Dolabellanin A

E. coli (LE-392) was incubated with dolabellanin A for 4 h (a) and *S. aureus* was incubated with dolabellanin A for 18 h (b).

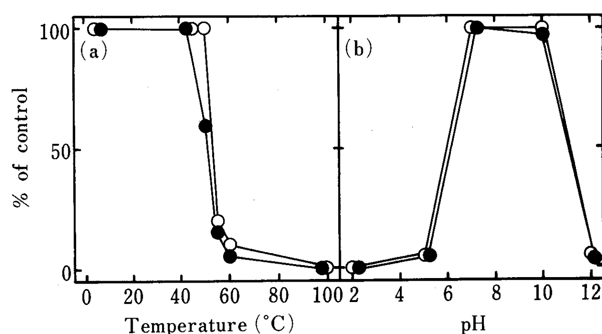

Fig. 2. Stability of Dolabellanin A at Various Temperatures and pHs

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

auricularia. About 16 mg of pure protein was obtained from 10 albumen glands. The specific activity of the purified material was increased about 10-fold over that of the crude homogenate, and on electrophoresis, the preparation gave a single band of 70 kilodaltons (kDa) (data not shown). These data suggest that the purity of dolabellanin A was nearly the same as that of the purified material obtained previously.[7]

This dolabellanin A inhibited the growth of *Escherichia coli* at a concentration of 0.1 μg protein/ml and that of *S. aureus* at 0.02 μg protein/ml (Fig. 1). As shown in Tables II and III, dolabellanin A inhibited the growth of all the bacteria tested, including both gram-positive and -negative strains. It therefore seems to be an antibacterial factor with a wider spectrum of activity than usual antibiotics or chemotherapeutic drugs.

Characterization of Dolabellanin A We first examined the stability of the antibacterial activity of dolabellanin A. As shown in Fig. 2A, the factor was heat-labile, showing appreciable loss of activity after heat-treatment at 55 °C for 10 min. Dolabellanin A was stable at neutral pH, but lost its activity at pH 12 and 5 (Fig. 2B). These treatments caused the loss of the antibacterial and antineoplastic activities

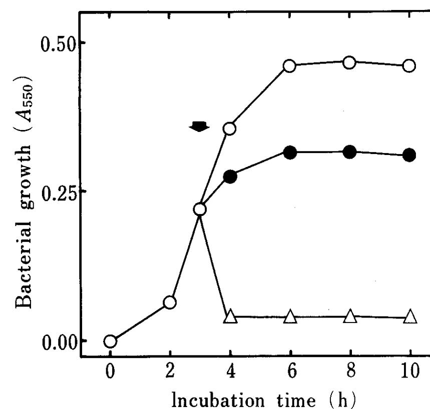

Fig. 3. Time Course of Antibacterial Effect of Dolabellanin A

E. coli (LE392) was incubated with dolabellanin A (●) or ampicillin (△), or without either (○). ↓: addition of dolabellanin A (10 μg/ml) or ampicillin (10 μg/ml).

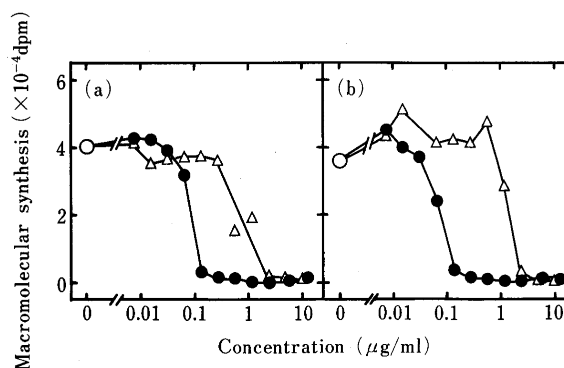

Fig. 4. Effects of Dolabellanin A on Synthesis of Nucleic Acids in *E. coli*

E. coli was incubated with or without dolabellanin A (●) or ampicillin (△), 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.

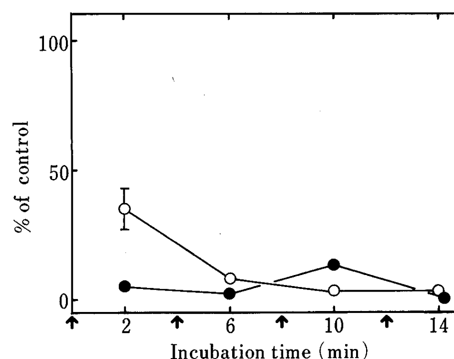

Fig. 5. Effects of Dolabellanin A on Pulse Labelling of Nucleic Acids

E. coli was incubated with or without dolabellanin A (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.

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	1 5 10 15	
	Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Gly-Lys-Phe-Gly-Lys-Ala-Phe-Val-	clawed frog[10]
	Lys-Trp-Lys-Val-Phe-Lys-Lys-Ile-Glu-Lys-Met-Gly-Arg-Asn-Ile-Arg-	cecropia moth[11]
	Ser-Trp-Leu-Ser-Lys-Thr-Arg-Lys-Lys-Leu-Glu-	pig[12]
	Gly-Trp-Leu-Lys-Lys-Ile-Gly-Lys-Lys-Ile-Glu-	flesh fly[13]
	Ser-Lys-Ser-Gly-Arg-Gln-Ile-( )-	sea hare[7]

Fig. 6. Amino Acid Sequences in N-Terminal Parts of Antimicrobial Peptides

simultaneously (Fig. 2A and 2B), suggesting that the active sites for the two activities are similar or identical.

To determine the antibacterial mechanism of dolabellanin A, we examined whether the factor showed bactericidal or bacteriostatic activity. As shown in Fig. 3, the growth of *E. coli* stopped after the addition of dolabellanin A, but the factor did not lyse the bacteria, in contrast to the bactericidal drug ampicillin. We also examined the correlation between cell metabolism and antibacterial activity. Figure 4 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 ampicillin. Thus, the growth inhibition by dolabellanin A may be due to the decrease in metabolic activities such as the syntheses of DNA and RNA. The results of a pulse experiment in Fig. 5 show that dolabellanin A completely inhibited the synthesis of nucleic acids in bacteria within 6 min.

Discussion

The Indian and Pacific Ocean sea hare, *Dolabella auricularia*, has been reported to contain cytotoxic substances of low molecular weight[8]; cytotoxic substances of high molecular weight, however, have not previously been reported except in our studies.[7,9] We reported that dolabellanin A was a 70-kDa glycoprotein consisting of four subunits.[7] This factor was half-maximally active at 1—18 ng protein/ml and lysed all the tumor cells tested, but did not lyse normal white or red blood cells.[9] Here, we purified dolabellanin A in a large quantity, examined its antibacterial action, and found that it inhibited the growth of all the bacterial strains tested at 18—480 ng protein/ml. Its action was not bactericidal, but bacteriostatic, so bacteria grew after dolabellanin A was removed. Dolabellanin A did not induce morphological elongation of bacteria (data not shown), suggesting that it did not inhibit cell wall synthesis. Moreover, it did not cause the release of adenosine triphosphate (ATP) from bacteria (data not shown). It did, however, inhibit the synthesis of DNA and RNA within a few minutes. Therefore, the antibacterial action of dolabellanin A 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 further study.

Most antitumor antibiotics also show both antibacterial and antineoplastic activities. Since dolabellanin A inhibited the synthesis of nucleic acids in tumor cells,[9] these two activities may share a common mechanism in terms of inhibition of nucleic acid synthesis. Moreover, these two activities were lost in parallel during various treatments (Fig. 2), suggesting that the active sites for the two activities may be similar or even identical.

Several antibacterial peptides have been reported recently,[10-13] and we compared dolabellanin A with those peptides. As shown in Fig. 6, similar sequences in N-terminal parts were observed among five antibacterial factors. It suggests that dolabellanin-like antibacterial peptides are common throughout the animal kingdom; for example, in a moth and a fly in arthropoda, a frog in amphibia and in a mammal, a pig. This broad distribution of antibacterial peptides in the animal kingdom indicates that these peptides have been well conserved during evolution, because animals cannot survive unless they eliminate invading bacteria.

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References

1) Part XII: J. Kisugi, H. Kamiya, and M. Yamazaki, *Chem. Pharm. Bull.*, **40**, 1641 (1992).
2) M. Yamazaki, "Immune Response," ed. by Y. Yamamura, Dobunshoin, Tokyo, 1986, pp. 653—667.
3) E. L. Cooper, *Endeavour*, **4**, 160 (1980).
4) P. J. Scheuer, "Chemistry of Marine Natural Products," Academic Press, New York, 1973, pp. 1—182.
5) I. Kitagawa, *Kagaku-Zhokan*, **111**, 133 (1987).
6) *a*) J. Kisugi, H. Kamiya, and M. Yamazaki, *Cancer Res.*, **47**, 5649 (1987); *b*) M. Yamazaki, K. Kimura, J. Kisugi, K. Muramoto, and H. Kamiya, *ibid.*, **49**, 3834 (1989); *c*) J. Kisugi, H. Kamiya, and M. Yamazaki, *Dev. Comp. Immunol.*, **13**, 3 (1989); *d*) M. Yamazaki, S. Tansho, J. Kisugi, K. Muramoto, and H. Kamiya, *Chem. Pharm. Bull.*, **37**, 2179 (1989); *e*) H. Kamiya, "Bioactive Marine Natural Products," ed. by T. Yasumoto and H. Kamiya, Kouseisha-Koseikaku, Tokyo, 1987, pp. 9—20; *f*) H. Kamiya, K. Muramoto, and M. Yamazaki, *Experientia*, **42**, 1065 (1986).
7) J. Kisugi, M. Yamazaki, Y. Ishii, S. Tansho, K. Muramoto, and H. Kamiya, *Chem. Pharm. Bull.*, **37**, 2773 (1989).
8) *a*) G. R. Pettit, R. H. Ode, C. L. Herald, R. B. von Dreele, and C. Michel, *J. Am. Chem. Soc.*, **98**, 4677 (1976); *b*) C. Ireland, D. J. Faulkner, J. Finer, and J. Clardy, *ibid.*, **98**, 4664 (1976); *c*) G. R. Pettit, Y. Kamano, C. L. Herald, A. A. Tuinman, F. E. Boettner, H. Kizu, J. M. Schmidt, L. Baczynsky, K. B. Tomer, and R. J. Bontems, *J. Am. Chem. Soc.*, **109**, 6883 (1987).
9) M. Yamazaki, J. Kisugi, and H. Kamiya, *Chem. Pharm. Bull.*, **37**, 3343 (1989).
10) M. Zasloff, *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 5449 (1987).
11) P. van Hofsten, I. Faye, K. Kockum, J. Y. Lee, K. G. Xanthopoulos, I. A. Boman, H. G. Boman, A. Engstrom, D. Andreu, and R. B. Merrifield, *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 2240 (1985).
12) J. Y. Lee, A. Boman, S. Chuanxin, M. Andersson, H. Jornvall, V. Mutt, and H. G. Boman, *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 9159 (1989).
13) M. Okada and S. Natori, *J. Biol. Chem.*, **260**, 7174 (1985).

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