Biopolymers from Marine Invertebrates. XI.[1] Characterization of an Antineoplastic Glycoprotein, Dolabellanin A, from the Albumen Gland of a Sea Hare, *Dolabella auricularia*

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An anti-neoplastic factor, dolabellanin A, inducing tumor lysis was purified from the albumen gland of a sea hare, *Dolabella auricularia*. Purified dolabellanin A was found to be a glycoprotein of 250 kilodaltons containing 4 subunits. This factor was half-maximally active towords a variety of tumor cells at 1—18 ng protein/ml and lysed tumor necrosis factor (TNF)-resistant tumor cells. Dolabellanin A was labile on heating, at low and high pH, and on treatment with urea, guanidine, sodium lauryl sulfate or trypsin, but not with 2-mercaptoethanol or periodate. Dolabellanin A completely inhibited the syntheses of deoxyribonucleic acid and ribonucleic acid by tumor cells within 1 h and caused their complete cytolysis within 18 h. Tumor lysis by dolabellanin A was not inhibited by anti-TNF antibody but was inhibited by certain sugars, suggesting that recognition of a sugar moiety is a key step in its induction of cytolysis. Dolabellanin A also prolonged the survival of mice bearing syngeneic MM46 ascitic tumors ($p<0.001$). These results suggest that dolabellanin A, found in an invertebrate, the sea hare, is a new antitumor factor.

Keywords antineoplastic factor; glycoprotein; cytolysis; tumor; sea hare; marine animal; *Dolabella auricularia*; biopolymer; albumen gland

Marine animals, which develop in a different environment from terrestial animals, have been reported to contain substances not found in terrestial animals.[2-4] We have also found new biologically active substances in marine animals.[5] Moreover marine invertebrates may contain special host defense factors, because their defense mechanisms differ from the immune systems of highly developed vertebrates.[6] With this idea in mind, we have searched for antibacterial and antitumor factors in sea hares of the species *Aplysia*.[7-11] Previously we reported a novel cytolytic factor, dolabellanin A, found in the albumen gland of *Dolabella auricularia* and its purification.[12] We showed that purified dolabellanin A was a glycoprotein of 250 kilodaltons (kDa) consisting of 4 subunits. We also determined the amino acid composition and the N-terminus of this factor. Here, we report the characterization of this novel cytolytic factor (dolabellanin A) and a comparison of some of its properties with those of cytolytic factors from *Aplysia* species.

Materials and Methods

Collection of *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.

Target Cells MM46, MM48 and MH134 tumor cells were collected from the peritoneal cavity of C3H/He mice. Human tumor cells, A549, LS 174T, T24 and WiDr, were harvested from *in vitro* cultures.

Cytolytic Assay Target tumor cells (2×10^6 cells/ml) were labeled with $Na_2{}^{51}CrO_4$ (100 μCi/ml) in RPMI medium containing 10% fetal calf serum (RPMI-FCS) for 2 h and then washed 3 times. Labeled target cells (5×10^3 cells) with or without a test preparation in wells (7 mm diameter) of flat-bottomed microplates were incubated in 0.2 ml of RPMI-FCS for 18 h at 37 °C under CO_2 in air. The radioactivity of the supernatant was measured and cytolytic activity was defined as follows:

$$\%\ \text{cytolysis}=\frac{\text{experimental count}-\text{control count}}{\text{maximum releasable count}-\text{control count}}\times100$$

Maximum release of ^{51}Cr was measured after freeze-thawing labeled tumor cells 3 times. The control count was measured as the radioactivity spontaneously released from labeled cells. Units of cytolytic activity were calculated as follows:

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

Cytolysis was also assessed by microscopic examination.

Assays of Synthetic Activities The metabolic activities of tumor cells with and without treatment with a cytolytic factor were measured in terms of incorporation of tritiated thymidine, uridine, and leucin into deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein, respectively. Samples of 1×10^5 MM46 tumor cells 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) (New England Nuclear, Boston, MA) in 0.2 ml of RPMI-fetal calf serum at 37 °C for 60 min. Then the cells were harvested on filters and washed with a Labomash LM-101. The filters were dried and their radioactivity was counted in a liquid scintillation spectrophotometer.

Tests of Statility Tests of stability were done as described previously.[9] Briefly, the pH stability of dolabellanin A was examined by adding 1 N HCl or 1 N NaOH to 1 ml samples to give the desired pH values. The samples were kept at 4 °C for 30 min and then readjusted to pH 7.0 and dialyzed against phosphate-buffered saline, pH 7.4. Other samples (1 ml) were treated with guanidine-HCl (6 M), sodium dodecyl sulfate (0.1%), 2-mercaptoethanol (0.1 M), or urea (8 M) at 37 °C for 2 h and then dialyzed. Samples were treated with periodate in the dark at 4 °C for 6 h and then dialyzed. Samples of 1 mg were incubated with 1 mg of trypsin (Difco) at 37 °C for 2 h.

Determination of Protein and Carbohydrate Amino acid composition and carbohydrate were analyzed as described previously.[9] Briefly, protein was hydrolyzed with 30 μl of 6 M HCl at 110 °C in evacuated tubes for 22 h and dried *in vacuo*. Amino acid derivatives were separated on a Hypersil ODS column (3 μm, 4.6 × 10 mm).

Results

Purification of the Antineoplastic Factor, Dolabellanin A The antineoplastic factor was purified from the albumen gland of the sea hare, *Dolabella auricularia*, by ion exchange chromatography and two types of gel filtration as reported previously.[12] Table I summarizes the purification of this cytolytic factor. About 50 mg of pure protein was obtained from 15 albumen glands. The specific activity was increased about 19-fold over that of the original homogenate and did not change appreciably when the preparation was fractionated further by high-performance liquid chromatography (HPLC) (LC-6A, Shimadzu) on a 0.75 × 60 cm column of G3000 SW (Toyo Soda Manufacturing Co.) (Fig. 1). The purified prparation gave a single protein band of 70 kDa on SDS-polyacrylamide gel electrophoresis with or without 2-mercaptoethanol (data

TABLE I. Purification of Dolabellanin A from Albumen Gland of a Sea Hare

Step	Activity (units $\times 10^{-4}$)	Protein (mg)	Specific activity (units/mg)	Purification fold	Yield (%)
Homogenate[a]	21	2200	95	1	100
DEAE-cellulose(I)	20	310	645	7	95
Sepharose 6B	15	110	1364	14	71
DEAE-cellulose(II)	9.4	51	1840	19	45

a) Fifteen albumen glands (18.7 g) were homogenized with 374 ml of 0.9% saline, and the homogenate was centrifuged to obtain a clear supernatant.

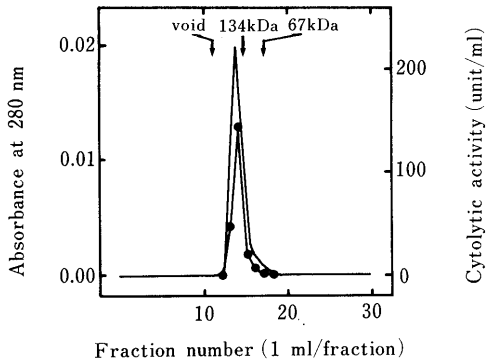

Fig. 1. Elution Pattern of the Cytolytic Factor (Dolabellanin A) on HPLC

The purified fraction from DEAE-cellulose (II) was subjected to HPLC on a 0.75 × 60 cm column of G3000 SW. Fractions (1 ml) were tested for cytolytic activity (●) and absorbance at 280 nm (——).

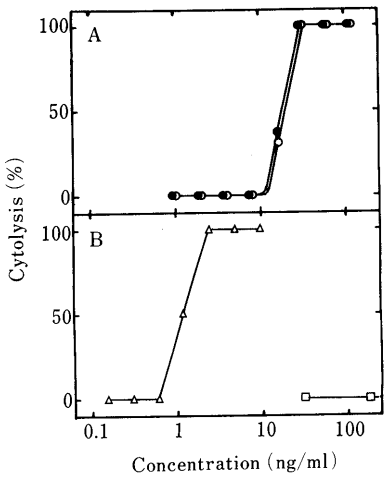

Fig. 2. Dose-Dependence of the Cytolytic Effect of Dolabellanin A

^{51}Cr-Labeled MM46 tumor cells (A) and T24 tumor cells (B) were incubated with dolabellanin A (○, △) or recombinant human TNF (□). Monoclonal anti-human TNF antibody (1 : 5000 dilution) was added during the cytolytic reaction (●).

not shown). These findings suggest that the purity of dolabellanin A was nearly the same as that of the purified material obtained previously.[12] We named this factor dolabellanin A, because it was purified from the albumen gland of *Dolabella*.

Dolabellanin A lysed murine MM46 tumor cells at a concentration of 20 ng protein/ml and its cytolytic activity was not inhibited by the addition of monoclonal anti-tumor necrosis factor (TNF) antibody (Fig. 2A). As shown in Fig. 2B, human T24 tumor cells were resistant to TNF but they were lysed by dolabellanin A at 2 ng protein/ml.

The kinetics of cytolysis was studied (Fig. 3). Tumor lysis

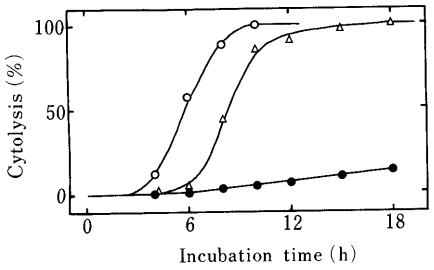

Fig. 3. Time Course of Tumor Lysis by Dolabellanin A

MM46 tumor cells were incubated with 0.2 μg/ml (△) or 3.5 μg/ml (○) of dolabellanin A, or without it (●).

TABLE II. Target Cell Specificity of Dolabellanin A

	Target cells	ED_{50}[a] (ng protein/ml)
Murine	MM46 (mammary carcinoma)	18
	MM48 (mammary carcinoma)	6
	MH134 (hepatoma)	2
	Macrophages	525
	Lymphocytes	1×10^5
	Red blood cells	$>2 \times 10^5$
Human	A549 (lung carcinoma)	1.2
	LS174T (colon carcinoma)	3.4
	T24 (bladder carcinoma)	1.2
	WiDr (colon carcinoma)	0.9
	TIG-1 (fetal lung)	62
	IMR-90 (fetal lung)	68
	WI-38 (lung diploid)	120
	Red blood cells	$>2 \times 10^5$

a) Target cells were incubated with dolabellanin A for 18 h. ED_{50}, concentration for 50% lysis of target cells.

TABLE III. Effects of Various Treatments on the Stability of Dolabellanin A

Treatment	Temperature (°C)	Time of treatment	Residual activity (%)
None			100
pH 4	4	30 min	< 1
pH 12	4	30 min	< 1
SDS (0.1%)	37	2 h	< 1
Urea (8 M)	37	2 h	< 1
Guanidine–HCl (6 M)	37	2 h	1.2
Heat	55	10 min	1.4
Trypsin (1 mg : 1 mg)	37	24 h	16
2-Mercaptoethanol (0.1 M)	37	2 h	39
Periodate (0.01 M)	4	6 h	53

by the cytolytic factor was time-dependent and was complete within 18 h. Tumor lysis was also examined by microscopy. After 6—8 h, the number of bubbles in target cells gradually increased, and after 10 h the cell surface membrane burst (data not shown).

Characterization of Dolabellanin A To determine the target specificity of dolabellanin A, we examined its cytolytic activity against various target cells. As shown in Table II, all the murine and human tumor cells tested were lysed by purified dolabellanin A in the concentration range of 1—18 ng protein/ml. In contrast, normal white and red blood cells were resistant to this cytolytic factor. These results indicate that tumor cells are relatively susceptible to dolabellanin A.

Table III summarizes the sensitivities of dolabellanin A to various treatments. Dolabellanin A was heat-labile,

showing appreciable loss of activity after heating at 55 °C for 10 min. It was stable at neutral pH (6—8), but lost its activity completely at pH 4 and 12. It also lost cytolytic activity on treatment with 8 M urea, 6 M HCl–guanidine, 0.1% SDS or trypsin, but was fairly resistant to treatment with 0.1 M 2-mercaptoethanol or 0.01 M periodate.

To determine the mechanism of cytolysis by dolabellanin A, we examined the correlation between cell metabolism and cytolysis. Figure 4 shows that dolabellanin A inhibited the abilities of tumor cells to incorporate thymidine and uridine within 1 h. It also inhibited protein synthesis within 3 h. Thus, decrease in metabolic activities, such as the syntheses of DNA, RNA and protein, may be followed by cytolysis.

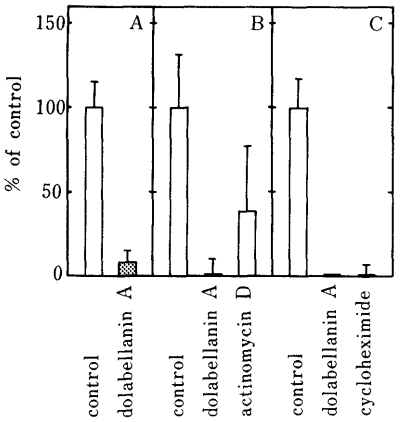

Fig. 4. Effects of Dolabellanin A on Syntheses of Macromolecules in Tumor Cells

MM46 tumor cells were incubated with or without dolabellanin A (40 ng/ml) and then with tritiated thymidine (A) or uridine (B) at 37 °C for 1 h. The incorporation of leucine at 37 °C for 3 h was also examined (C). Positive controls contained actinomycin D and cycloheximide at 1 and 5 μg/ml, respectively. The acid-insoluble fraction was obtained and its radioactivity was measured. Bars, S.D.

$$\text{percent of control} = \frac{\text{dpm of factor-treated cells}}{\text{dpm of untreated cells}} \times 100$$

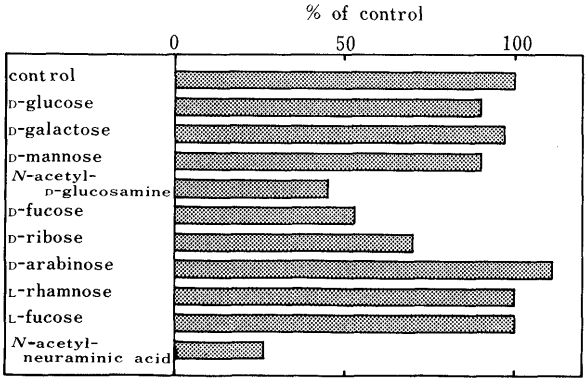

Fig. 5. Effects of Sugars on Cytolysis by Dolabellanin A

^{51}Cr-Labeled MM46 tumor cells and 40 ng of dolabellanin A/ml were incubated at 37 °C for 18 h with or without the indicated sugars at final concentrations of 50 mM.

To determine the mechanism of cell recognition by dolabellanin A, we examined the effects of various sugars on the cytolysis. As shown in Fig. 5, some sugars at concentrations of 50 mM inhibited tumor lysis by dolabellanin A. Thus, recognition of specific sugars such as *N*-acetylneuraminic acid, *N*-acetyl-D-glucosamine and fucose may be an initial step in cytolysis induced by dolabellanin A. However, we do not yet know whether these sugars that are essential for cytolysis are present on the tumor cells or on dolabellanin A.

We tested the *in vivo* antitumor activity of dolabellanin A by incubating mixtures of tumor cells and dolabellanin A *in vitro* and then injecting them intraperitoneally into mice. Mitomycin C was used as a positive control. Table IV (expt. 1) shows that dolabellanin A had a protective effect against the MM46 tumor ($p<0.001$). Some mice that survived were resistant to challenge with a lethal dose of fresh tumor cells. Next, to test the therapeutic effect of dolabellanin A, we injected it intraperitoneally into mice after inoculation of MM46 tumor cells into the peritoneal cavity. Table IV (expt. II) shows that some animals survived after injection of 4 μg/mouse daily for 6 d.

Comparison of Properties of Dolabellanin A and Aplysianins E and A We previously isolated two cytolytic factors, aplysianin E from the eggs[9] and aplysianin A from

Table IV. Effect of Dolabellanin A on Survival of Mice Bearing Ascitic MM46 Tumor Cells

Treatment	Dose/mouse	Survival ratio	Survival days
Expt. I (Neutralization)[a]			
Saline		0/5	16.8±0.8
Mitomycin C	10 μg	5/5	>50
Dolabellanin A	0.2 μg	0/5	17.8±0.8
Dolabellanin A	2 μg	0/5	19.2±1.6 $p<0.05$
Dolabellanin A	20 μg	3/5	22.5±2.1 $p<0.001$
Expt. II (i.p.injection)[b]			
Saline	0.2 ml×6	0/6	16.3±1.0
Mitomycin C	10 μg×6	5/5	>50
Dolabellanin A	0.8 μg×6	0/6	17.3±1.4
Dolabellanin A	4 μg×6	3/6	20.3±4.2 $p<0.001$

a) Murine MM46 tumor cells were treated with mitomycin C or dolabellanin A *in vitro* at 37 °C for 30 min. Then the cells (5×10^4 cells/mouse) were injected i.p. into C3H/He mice. *b*) C3H/He mice received ascitic MM46 cells i.p. on day 0 and were treated i.p. with saline, mitomycin C or dolabellanin A daily for 6 d from day 0.

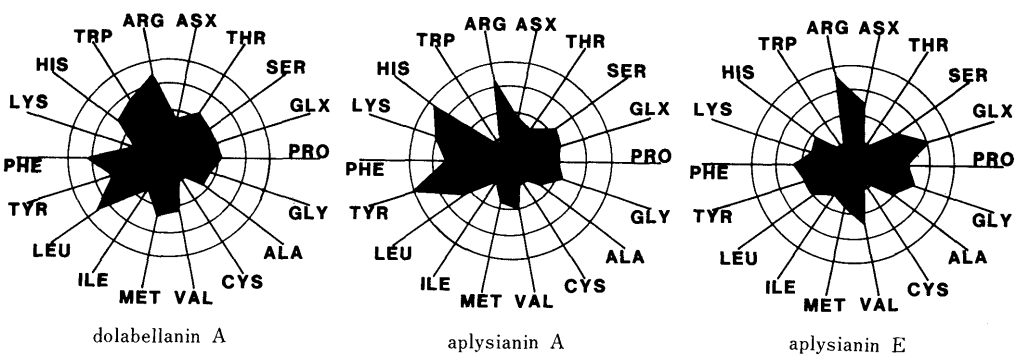

Fig. 6. Graphic Presentation of Amino Acid Compositions of Cytolytic Proteins with a Modified Radar Chart

TABLE V. Comparison of Properties of Aplysianins A and E and Dolabellanin A

Property	Dolabellanin A from albumen gland of *D. auricularia*	Aplysianin A from albumen gland of *A. kurodai*	Aplysianin E from eggs of *A. kurodai*
Molecular weight (kDa)	250	320	250
Subunit	4 (70 kDa × 4)	4 (85 kDa × 4)	3 (76, 88, 102 kDa)
Sugar content (%)	10.9	9.8	8
Concentration of NaCl for elution (mM)	60	75	75
Amino acid composition	Leu, Glx, Asx rich	Glx, Asx rich	Glx, Asx rich
Reaction with anti-dolabellanin A serum (Ouchterlony)	+	−	−
Reaction with anti-aplysianin E serum (Ouchterlony)	−	±(with spur)	+
Reaction with anti-TNF antibody	−	−	−
Time for cytolysis (h)	18	18	18
Heat treatment (55 °C, 10 min)	Labile	Labile	Labile

the albumen gland[8] of *Aplysia kurodai*. We compared the amino acid composition of dolabellanin A with those of aplysianins E and A by the method of Nakamura and Furukohri.[13] As shown in Fig. 6, the amino acid composition of dolabellanin A was not similar to that of aplysianin E or A, but the compositions of the latter two were similar, particularly as regards lysine and tryptophan. Some properties of dolabellanin A and aplysianins E and A are listed in Table V.

Discussion

Marine animals are good sources of new biologically active substances, because they develop in a different environment from terrestial animals.[2-5] A sea hare of the Pacific and Indian Oceans, *Dolabella auricularia*, has been found to contain biologically active substances of low molecular weight,[14-16] but cytotoxic substances of high molecular weight have not yet been isolated from it. Previously, we reported the presence of a novel cytolytic factor, dolabellanin A, in the albumen gland of *D. auricularia* and its purification.[12] We found that purified dolabellanin A was a glycoprotein of 250 kDa consisting of 4 subunits. We also determined the amino acid composition and the N-terminus of the factor. We have now purified dolabellanin A on a large scale and examined its characteristics and its cytolytic action.

Dolabellanin A lysed a variety of tumor cells, including TNF-resistant tumor cells, and its cytolytic activity was not inhibited by anti-TNF antibody. Therefore, its active site for cytolysis may be different from that of TNF.

Dolabellanin A inhibited the syntheses of macromolecules such as DNA, RNA and protein before causing cytolysis (Figs. 3 and 4). Furthermore, its cytolytic effect was temperature-dependent (data not shown). These observations suggest that its cytolytic mechanism is closely related to cellular metabolism. In other words, dolabellanin A does not act on target cells as a detergent. Target cells may have a receptor for dolabellanin A, as its cytolytic effect was inhibited by certain sugars (Fig. 5). Therefore, dolabellanin A may have two characteristics favorable for killing tumor cells: an antiproliferative effect, like an anticancer drug, and the ability to recognize target cells selectively.

We have purified two antitumor factors from *Aplysia kurodai*, aplysianin E from the eggs[9] and aplysianin A from the albumen gland.[8] Aplysianin E has a molecular weight of 250 kDa and is composed of three distinct subunits with molecular weights of 76, 88 and 102 kDa. Aplysianin A, which has a moleclar weight of approximately 320 kDa and consists of four 85 kDa subunits, is larger than aplysianin E. These two factors give similar graphic presentations of amino acid compositions in a modified radar chart (Fig. 6) and have a common epitope (Table V), suggesting that aplysianin A is a precusor of aplysianin E. As shown in Table V, dolabellanin A did not resemble these two antitumor factors in antigenicity or amino acid composition, although these three factors showed similar kinetics of cytolysis, a similar tumor spectrum and similar sensitivity to various treatments. These results suggest that dolabellanin A differs from aplysianin A and E, although its partial structure responsible for cytolysis may be similar to those of aplysianins A and E.

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