Purification and Characterization of a Cytolytic Protein from Purple Fluid of the Sea Hare, *Dolabella auricularia*

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A novel cytolytic factor, dolabellanin P, was purified to apparent homogeneity from the purple fluid of the sea hare, *Dolabella auricularia*. Purified dolabellanin P is a single polypeptide of 60 kDa. The amino acid composition and the N-terminus of the factor were also determined. This factor nonspecifically lysed all the cells tested at 50—200 ng protein/ml. Dolabellanin P caused complete cytolysis within 2 h. The factor is distinct from antineoplastic glycoproteins previously isolated from eggs (aplysianin E) or albumen gland (aplysianin A) of *Aplysia kurodai* in terms of certain cytolytic properties. These results suggest that dolabellanin P, found in the sea hare, a marine invertebrate, is a new cytolytic factor.

Keywords cytolytic factor; sea hare; tumor; dolabellanin P; *Dolabella auricularia*

The Indian and the Pacific Ocean *Dolabella* species of sea hares have attracted the interest of many researchers investigating biologically active substances.[1] These substances are low-molecular-weight compounds and most of them are derived from algal diets.[2] *Dolabella* species discharge a purple fluid from a purple gland when disturbed. It seems likely that some bioactive factors which take part in the host defense mechanisms may be contained in the purple fluid of this species. No bioactive substance has heretofore been isolated from the fluid, but here, we report the isolation and characterization of a novel cytolytic protein, dolabellanin P, from the purple fluid of *Dolabella auricularia*.

Materials and Methods

Collection of Purple Fluid from *Dolabella auricularia* Specimens of *D. auricularia* were collected in Tateyama, Kominato, Aburatsubo and Kochi, Japan, in May, June and July. The purple fluid was obtained by agitating the animals and was frozen at −80 °C until use.

Target Cells MM46, MH134 and MM48 tumor cells were collected from the peritoneal cavity of C3H/He mice. Human tumor cells, A549, T24 and SK LEU-1, 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:

$$\% \text{ cytolysis} = \frac{\text{experimental count} - \text{control count}}{\text{maximum releasable count} - \text{control count}} \times 100$$

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:

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

Polyacrylamide Gel Electrophoresis Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis was performed by the method of Laemmli in 12.5% acrylamide gels.[3] Samples were heated at 100 °C for 3 min in 1% SDS in the presence of 2% 2-mercaptoethanol. The gels were stained with Coomassie brilliant blue.

Amino Acid Analysis Amino acid composition was analyzed by the method of Chang *et al.*[4] Protein (4 μg) was hydrolyzed with 30 μl of 6 M HCl at 110 °C in evacuated tubes for 22 h and the hydrolysate was evaporated *in vacuo*. The hydrolysate was derivatized with 4-dimethylaminoazobenzene-4′-sulfonyl chloride and diluted with 10 mM sodium phosphate buffer (pH 6.5). Amino acid derivatives were separated on a Hypersil ODS column (3 μm, 4.6 × 10 mm).

The amino acid sequence of a cytolytic factor was determined at the Toray Research Center (Tokyo, Japan) by an automated Edman degradation process using a protein sequencer (Applied Biosystems 470A and 120A).

Results

Purification of Dolabellanin P from Purple Fluid The purple fluid of *D. auricularia* was centrifuged at 10000 rpm for 30 min before use. The supernatant of the fluid (946 ml) was precipitated with ammonium sulfate at a final concentration of 25% to remove purple dye. The precipitates were dissolved in 150 mM NaCl solution, and the solution was dialyzed against 10 mM phosphate buffer containing 150 mM NaCl and loaded onto a column (3.2 × 40 cm) of DE52 (Whatman, Maidstone, U.S.A.) previously equilibrated with the starting buffer (150 mM NaCl containing 10 mM phosphate buffer, pH 7.4). Cytolytic activity was recovered from the flow-through fractions (Fig. 1A). The colorless fractions with the activity were combined, concentrated on an ultrafiltration membrane (Toyo Kagaku, Tokyo, Japan) and applied to a column of Sepharose 6B (2.6 × 109 cm) (Fig. 1B). Pooled fractions were applied to a column (0.5 × 6 cm) of DE52 (data not shown) and the flow-through fractions with cytolytic activity were combined. The cytolytic activity was finally eluted with 150 mM NaCl containing 10 mM phosphate buffer, pH 7.4, on a column (0.5 × 6 cm) of CM52 (Whatman, Maidstone, U.S.A.) (Fig. 1C).

Table I summarizes the purification of the cytolytic factor, dolabellanin P. About 310 μg of pure protein was obtained from 946 ml of purple fluid. The specific activity was increased about 300-fold compared with the original purple fluid and did not change appreciably when this fraction was further fractionated by high-performance liquid chromatography (LC-6A, Shimadzu) with a 0.75 × 60 cm column of G3000 SW (Toyo Soda Manufacturing Co.). The purified factor contained 0.8% sugar, suggesting that it is a glycoprotein.

To examine the subunit structure of the cytolytic factor, we subjected the purified dolabellanin P to SDS–polyacrylamide gel electrophoresis. Figure 2 shows that the purified preparation gave a single protein band of 60 kDa

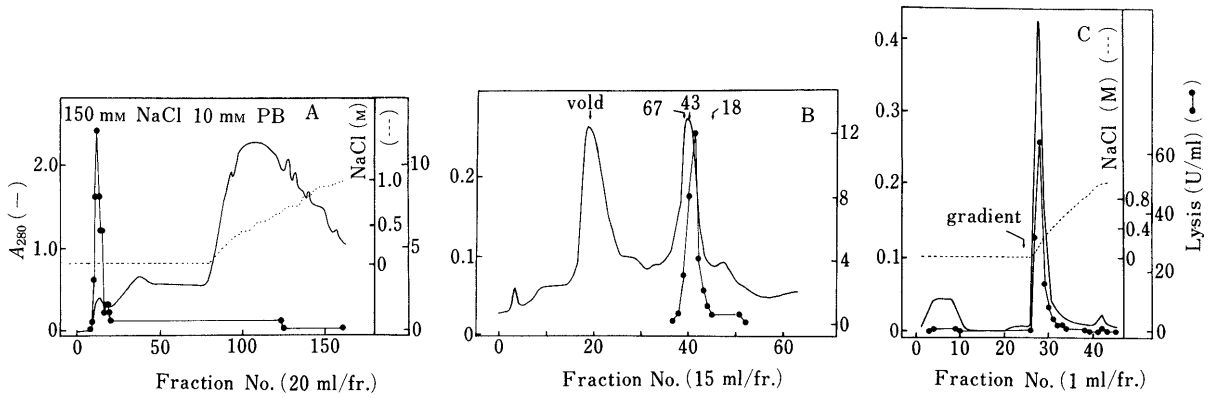

Fig. 1. Elution Patterns of the Cytolytic Factor (Dolabellanin P) on Column Chromatography

The concentrated purple fluid was applied to a diethylaminoethyl (DEAE) cellulose column I (3.2 × 40 cm) (A) and the active fraction from this column was applied to a Sepharose 6B column (2.6 × 109 cm) (B). The active fraction from the Sepharose 6B column was then applied to a DEAE cellulose column II (0.5 × 6 cm) and the active fraction from this column was purified on a carboxymethyl (CM) cellulose column (0.5 × 6 cm) (C). Fractions were tested for cytolytic activity (●), NaCl concentration (------) and absorbance at 280 nm (——). Myoglobin (18.8 kDa), ovalbumin (43 kDa) and bovine serum albumin (67 kDa) were used as marker proteins.

TABLE I. Purification of Dolabellanin P from Purple Fluid of *Dolabella auricularia*

	Volume (ml)	Activity (units)	Protein[a] (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude fluid	946	662	2460	0.27	1	100
DEAE-cellulose (I)	7.3	277	88	3.1	11	42
Sepharose-6B	7.9	158	4.8	33	122	24
DEAE-cellulose (II)	7.7	165	2.5	66	244	25
CM-cellulose	1.0	25	0.31	81	300	3.8

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

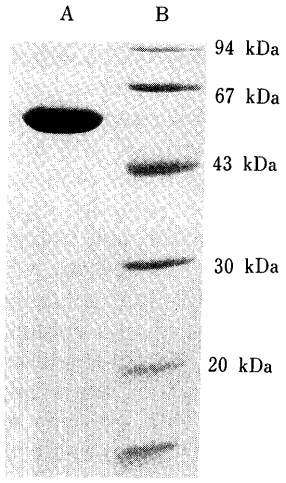

Fig. 2. SDS–Polyacrylamide Gel Electrophoresis of Purified Dolabellanin P

The purified sample was subjected to electrophoresis on 12.5% acrylamide slab gel at a constant voltage of 120 V for 3 h. (A) The purified sample (20 μg protein). (B) Markers (phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa).

on SDS–polyacrylamide gel electrophoresis. The presence of 2-mercaptoethanol did not affect the molecular mass of the 60 kDa component. These results indicate that the cytolytic factor, dolabellanin P is a 60 kDa glycoprotein containing a single polypeptide.

Characterization of Dolabellanin P The kinetics of cytolysis was studied (Fig. 3). Cytolysis by dolabellanin P was time-dependent and was complete within 2 h. Cytolysis was also examined by microscopy. After 30 min, the number of bubbles in MM46 target cells gradually increased, and after

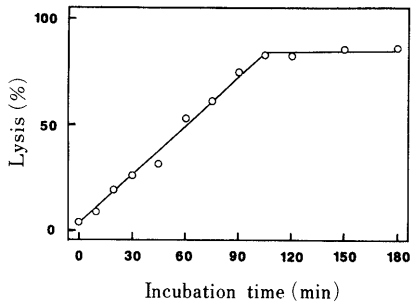

Fig. 3. Time Course of Tumor Lysis by Dolabellanin P

^{51}Cr-labeled MM46 tumor cells were incubated with dolabellanin P (0.5 μg protein/ml) for 3 h. Samples of supernatant were taken at the times indicated for measurement of radioactivity.

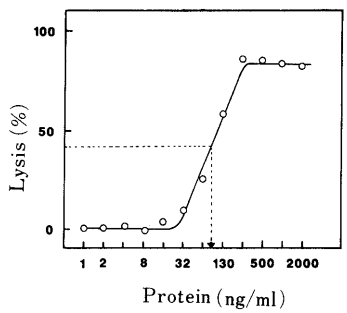

Fig. 4. Dose-Dependence of the Cytolytic Effect of Dolabellanin P

^{51}Cr-labeled MM46 tumor cells were incubated with dolabellanin P for 18 h. Cytolysis was measured as the radioactivity of the supernatant.

1 h the cell surface membrane burst (data not shown).

The preparation of dolabellanin P lysed murine MM46 tumor cells at a concentration of 100 ng protein/ml (Fig. 4).

We then examined the cytolytic activity against various target cells. As shown in Table II, all the tumor and normal cells tested were lysed by purified dolabellanin P in the concentration range of 50—200 ng protein/ml. These results indicate that dolabellanin P in the purple fluid is a non-specific cytotoxic substance.

Examination of the amino acid composition of dolabellanin P revealed that it contained a relatively large amount of Asx and Glx (Table III). The sequence of the amino-terminal region of dolabellanin P is shown in Fig. 5.

$$\overset{1}{\text{Val}}\text{–(}\ \text{)–Arg–Clu–}\overset{5}{\text{Asp}}\text{–Gln–Lys–Trp–}$$

$$\overset{10}{\text{Lys}}\text{–Leu–Ser–Phe–Asp–Lys–}\overset{15}{\text{Glu}}\text{------}$$

Fig. 5. Amino-Terminal Sequence of Dolabellanin P

TABLE II. Target Cell Specificity of Dolabellanin P

	Target cells	ED_{50}[a] (ng protein/ml)
Murine	MM46 (mammary adenocarcinoma)	100
	MM48 (mammary adenocarcinoma)	80
	MH134 (lung adenocarcinoma)	60
Human	A 549 (lung adenocarcinoma)	100
	T 24 (urinary bladder carcinoma)	50
	SK LEU-1 (lung adenocarcinoma)	200
Murine	Spleen cells	67
	Thymus cells	150

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

TABLE III. Amino Acid Composition of Dolabellanin P and Sugar

Amino acid	Composition (mol%)	Amino acid	Composition (mol%)
Ala	7.5	Phe	4.0
Arg	5.6	Pro	5.3
Asx	12.0	Ser	9.2
Cys	0	Thr	8.1
Glx	10.6	Trp	0.8
Gly	7.3	Tyr	4.1
His	4.8	Val	4.5
Ile	3.3	Sugar[a]	
Leu	6.0	Mannose	0.5[b]
Lys	6.1	Fucose	0.3[b]
Met	2.2		

a) Sugar was measured by the procedure of Muramoto *et al.*[10] *b*) Weight %.

Discussion

The Indian and the Pacific Ocean sea hare, *Dolabella auricularia*, is a large shell-less opisthobranch mollusc which is found intertidally feeding on brown algae. *Dolabella* species have been reported to contain low-molecular-weight cytotoxic substances[1]; however, cytotoxic substances of high-molecular-weight have not yet been reported.

Dolabella species discharge a purple fluid from a purple gland when disturbed. It seems likely that the fluid may contain bioactive components which participate in the host defense mechanism. Although no bioactive substance had previously been isolated from this fluid, we purified a novel cytolytic factor (dolabellanin P) from the purple fluid of *Dolabella auricularia* to a homogeneous species of 60 kDa. This factor is distinct from the purple dye previously reported[5] and seems to be a single polypeptide with 0.8% sugar moiety.

We previously found high-molecular-weight compounds with antineoplastic and antibacterial activity in the eggs,[6] purple fluid,[7] and albumen gland[8] of another sea hare, *Aplysia kurodai*. The active substances of *A. kurodai* were isolated as three type of glycoproteins.[6-8] As shown in Table IV, aplysianin E from *A. kurodai* eggs is a glycoprotein of 250 kDa having three distinct subunits of 76, 88 and 102 kDa. Aplysianin A from *A. kurodai* albumen gland is a glycoprotein of approximately 320 kDa and contains four 85 kDa subunits, being larger than aplysianin E. These two factors have a common antigen and a similar amino acid composition, suggesting that aplysianin A is a precursor of aplysianin E.[8]

Aplysianin P from the purple fluid does not resemble these two antineoplastic glycoproteins in terms of antigenicity or amino acid composition, although all three factors have a similar kinetics of cytolysis, a similar antitumor spectrum and a similar sensitivity to various treatments. In contrast, dolabellanin P, the nonspecific cytolytic factor in the purple fluid of *Dolabella auricularia*, differs from the antineoplastic factors of *Aplysia kurodai* in its cytolytic properties (Table IV).

The physiological function of dolabellanin P is not known at present. The purple gland of *Dolabella auricularia* is located under the gills, so that dolabellanin P, when its release is stimulated, may take part in cytolysis and/or act to eliminate small organisms in gills of this species. Dolabellanin P may be one component of a host defense system. Further studies are required on this point.

TABLE IV. Comparison of Properties of Cytolytic Glycoproteins

Origin	Name	Molecular weight (kDa)	Subunit	Sugar content (%)	Amino acid composition	Cytolysis completion (h)	Cytolysis at 15 °C	Target specificity
Purple fluid of *D. auricularia*	Dolabellanin P	60	1	0.8	Asx, Glx, Ser rich	2	(+) Positive	Nonspecific
Purple fluid of *A. kurodai*	Aplysianin P[a]	60	1	18	Pro, Gly, Thr rich	18	(−) Negative	Highly cytotoxic to tumor cells
Eggs of *A. kurodai*	Aplysianin E[b]	250	3 (76, 88, 102 kDa)	8	Glx, Asx, Gly rich	18	(−) Negative	Highly cytotoxic to tumor cells
Albumen gland of *A. kurodai*	Aplysianin A[c]	320	4 (85 kDa 4)	9.8	Glx, Asx, Lys rich	18	(−) Negative	Highly cytotoxic to tumor cells

a) Yamazaki *et al.*, 1986.[7] *b*) Kisugi *et al.*, 1987.[6] *c*) Kamiya *et al.*, 1986.[8]

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