Purification of a Novel Cytolytic Protein from Albumen Gland of the Sea Hare, *Dolabella auricularia*

Jun KISUGI,[a] Masatoshi YAMAZAKI,*[,a] Yasuhiro ISHII,[a,b] Shigeru TANSHO,[a] Koji MURAMOTO[c] and Hisao KAMIYA[c]

Faculty of Pharmaceutical Sciences, Teikyo University,[a] Sagamiko-cho, Tsukui-gun, Kanagawa 199-01, Japan, Research Institute, Kotobuki Seiyaku Co., Ltd.,[b] 6351 Sakaki, Nagano 389-06, Japan and School of Fisheries Sciences, Kitasato University,[c] Sanriku, Iwate 022-02, Japan.
Received March 16, 1989

A novel cytolytic factor, dolabellanin A, was purified to apparent homogeneity from the albumen gland of the sea hare *Dolabella auricularia*. Purified dolabellanin A was a glycoprotein of 250 kilo daltons containing 4 subunits. The amino acid composition and the N-terminal amino acid sequence of the factor were also determined. The factor is distinct from antineoplastic glycoproteins previously isolated from another sea hare, *Aplysia kurodai*. These results suggest that dolabellanin A found in the sea hare of the *Dolabella* species is a new cytolytic factor.

Keywords tumor lysis; cytolytic factor; glycoprotein; sea hare; *Dolabella auricularia*; albumen gland; cytolysis

The Indian and Pacific Ocean sea hare, *Dolabella auricularia*, is a large shell-less opisthobranch mollusk which is found intertidally feeding on brown algae. Some of the *Dolabella* species have attracted the interest of researchers investigating low-molecular-weight biologically active substances,[1-3] which are mostly derived from dietary algae. We previously found high-molecular-weight compounds with antineoplastic and antibacterial activity in sea hares, *Aplysia kurodai* and *A. juliana*.[4-7] Recently we have found that the potent cytolytic factor in the albumen gland of *D. auricularia* differs from that in *Aplysia* species. In this report, we describe the purification of the novel cytolytic glycoprotein, dolabellanin A, from the albumen gland of *D. auricularia*.

Materials and Methods

D. 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.

Cytolytic Activity Assay MM46 tumor cells were collected from the peritoneal cavity of C3H/He mice. Target MM46 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 with RPMI-FCS. 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 released spontaneously from labeled cells. Units of cytolytic activity were calculated as follows:

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

Cytolytic assay was also microscopically performed.

Polyacrylamide Gel Electrophoresis Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis was performed by the method of Laemmli[8] in 12.5% acrylamide gels. 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. Gradient gel electrophoresis was performed in 4—30% polyacrylamide gel.

Amino Acid Analysis Amino acid composition was analyzed by the method of Chang *et al.*[9] Protein was hydrolyzed with 30 μl of 6 N HCl at 110 °C in evacuated tubes for 22 h and dried *in vacuo*. The hydrolyzate 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 partially determined at the Toray Research Center (Tokyo, Japan) by an automated Edman degradation process using a protein sequencer (Applied Biosystems 470A and 120A).

Determination of Protein and Carbohydrate Protein was measured by the procedure of Lowry *et al.*[10] with bovine serum albumin as a standard. Analysis of reducing sugars was done using chromophoric hydrazones.[11]

Results

Purification of a Cytolytic Factor, Dolabellanin A We initially checked which organs of the sea hare, *D. auricularia*, showed cytolytic activity. Each organ was homogenized with 2—20 volumes of 0.9% saline for 10 min, and the homogenate was centrifuged at 10000 rpm for 30 min. The supernatant was used for cytolytic activity assay. As shown in Table I, the albumen gland complex had strong cytolytic activity. The albumen gland complex of *D. auricularia* visually consists of 3 parts, among which the albumen gland showed high specific and total activity (Table I). We therefore selected this part of the albumen gland complex as the starting material for purification of the cytolytic factor. Use of this material enabled us to easily purify the cytolytic factor, dolabellanin A.

The cytolytic factor was purified by ion exchange chromatography and two types of gel filtration. The supernatant of the homogenate (175 ml) of 11 albumen glands was dialyzed against 10 mM phosphate buffer and loaded onto a column (3.2 × 40 cm) of DE52 (Whatman,

TABLE I. Cytolytic Activities of Various Organs

Organ[a]	ED_{50} (unit/ml)	Total activity (unit/animal)
Spermatic sac	0.04	2
Generative gland	0.06	5
Oviduct	0.12	4
Digestive gland	0.32	96
Stomach	0.33	10
Gill	2.0	50
Coelomic	2.5	121
Purple fluid	4.1	28
Albumen gland complex (Expt. 1)	446.7	4315
Albumen gland complex (Expt. 2)		
(Albumen gland)	226.0	2848
(Spiral gland)	5.9	38
(Mucous gland)	17.8	413

a) Each organ was homogenized with 0.9% saline, and the homogenate was centrifuged to obtain a clear supernatant.

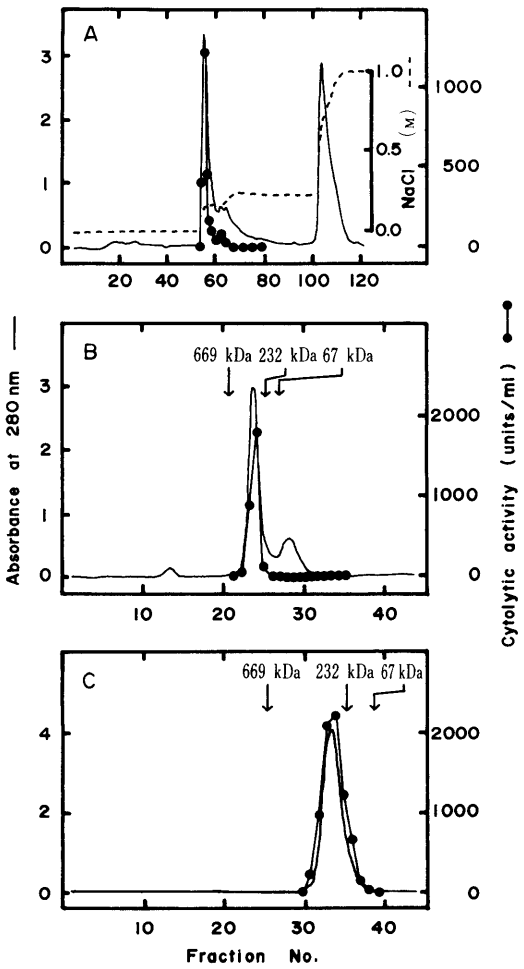

Fig. 1. Elution Patterns of the Cytolytic Factor (Dolabellanin A) on Column Chromatographies

The dialyzed homogenate was applied to a DEAE cellulose column (A) and the active fraction from this column was applied to a Sepharose 6B column (B). The active fraction from the Sepharose 6B column was then applied to a Sephacryl S-300 column (C). Fractions (15 ml) were tested for cytolytic activity (●), NaCl concentration (------) and absorbance at 280 nm (——). Bovine serum albumin (67 kDa), catalase (232 kDa) and thyroglobulin (669 kDa) were used as marker proteins.

Table II. Purification of Dolabellanin A from Albumen Gland of *D. auricularia*

Step	Activity (units $\times 10^{-3}$)	Protein (mg)	Specific activity (units/mg)	Purification fold	Yield (%)
Homogenate[a)]	110	1122	98	1	100
DEAE-cellulose	60	100	600	6	50
Sepharose 6B	42	38	1140	11	39
Sephacryl S300	25	25	1000	10	23

a) Eleven albumen glands (5.8 g) were homogenized with 116 ml of 0.9% saline, and the homogenate was centrifuged to obtain a clear supernatant.

Maidstone) previously equilibrated with the starting buffer (10 mM phosphate, pH 7.4). This column was washed with the starting buffer and then material was eluted with 150 mM NaCl solution (Fig. 1A). The fractions with activity were combined (80 ml), concentrated on an ultrafiltration membrane (Toyo Kagaku, Tokyo) and then applied to a column of Sepharose 6B (2.6 × 109 cm) (Fig. 1B). Fractions containing cytolytic factor were pooled, concentrated and applied to a column (1.8 × 106 cm) of Sephacryl S-300

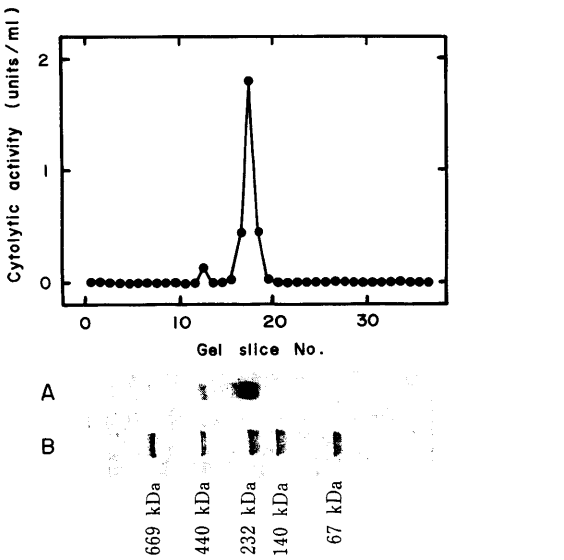

Fig. 2. Gradient Gel Electrophoresis of Purified Factor

The purified factor was subjected to electrophoresis on 4—30% acrylamide slab gel at a constant voltage of 125 V for 15 h. (A) Purified factor (6 μg protein). (B) Molecular mass markers (thyroglobulin 669 kDa, ferritin 440 kDa, catalase 232 kDa, lactate dehydrogenase 140 kDa, bovine serum albumin 67 kDa). The cytolytic activity (●) was determined for each fraction eluted from 2 mm sections of the gel.

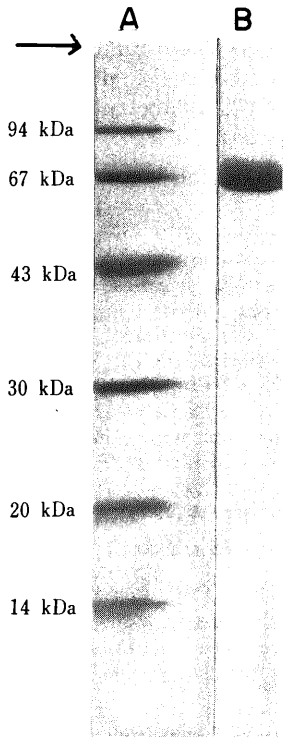

Fig. 3. SDS–Polyacrylamide Gel Electrophoresis of Purified Factor

The purified sample was subjected to electrophoresis on 12.5% acrylamide slab gel at a constant voltage of 120 V for 3 h. (A) Markers (phosphorylase b 94 kDa, bovine serum albumin 67 kDa, ovalbumin 43 kDa, carbonic anhydrase 30 kDa, soybean trypsin inhibitor 20 kDa, α-lactalbumin 14 kDa). (B) Purified sample (4 μg protein).

(Pharmacia, Uppsala, Sweden) in phosphate buffer (Fig. 1C). The activity appeared in fractions corresponding to a molecular weight of about 250 kilo daltons (kDa).

Table II summarizes the purification of the cytolytic factor. About 25 mg of pure protein was obtained from 11 albumen glands. The specific activity was increased about 10 fold over the starting homogenate and did not change

appreciably when this fraction was fractionated further by high-performance liquid chromatography (LC-6A, Shimadzu) on a 0.75 × 60 cm column of G3000 SW (Toyo Soda Manufacturing Co.) (data not shown). We called this factor dolabellanin A, since it was purified from the albumen gland of the *Dolabella* species.

To confirm that the purified factor was associated with the cytolytic activity, we subjected it to electrophoresis on 4—30% polyacrylamide gel (Pharmacia, Uppsala, Sweden) and examined the cytolytic activity of slices of the gel. As shown in Fig. 2, the purified factor gave a main band of 250 kDa and a minor band of 450 kDa. The position of cytolytic activity coincided with that of the main protein band. The minor band may be aggregated dolabellanin A formed during storage.

To examine the subunit structure of the cytolytic factor, we subjected the purified dolabellanin A to SDS–polyacrylamide gel electrophoresis. Figure 3 shows that the purified preparation gave a band of 70 kDa. Treatment of the factor with 2-mercaptoethanol did not affect the molecular size of this component. These results indicate that the cytolytic factor, dolabellanin A, is a protein of 250 kDa containing 4 subunits.

Properties of Dolabellanin A Examination of the amino acid composition of dolabellanin A revealed that it contained relatively large amount of leucine (Leu), glutaminic acid plus glutamine (Glx) and aspartic acid plus asparagine (Asx) (Table III). It also contained 11% sugar, suggesting that it was a glycoprotein (Table III). The sequence of the amino-terminal region of dolabellanin A is shown in Fig. 4.

We have also isolated a nonspecific cytolytic factor, dolabellanin P from the purple fluid of *D. auricularia* (manuscript in preparation). The amino acid composition of dolabellanin A was compared with that of dolabellanin P by the method of Nakamura and Furukohri[12] (Fig. 5). Properties of dolabellanins A and P are also listed in Table IV.

TABLE III. Amino Acid and Sugar Compositions of Dolabellanin A

Amino acid	Composition (mol%)
Ala	7.6
Arg	7.5
Asx	9.2
Cys	0
Glx	10.1
Gly	7.0
His	2.5
Ile	3.8
Leu	11.9
Lys	4.2
Met	2.3
Phe	5.3
Pro	4.5
Ser	6.5
Thr	6.2
Trp	1.7
Tyr	3.4
Val	7.4
Sugar	**Composition (weight%)**
Mannose	5.4
Galactose	3.6
Fucose	1.4
Xylose	0.5

NH_2–(Ser)^{1}–Lys–Ser–Gly–Arg^{5}–Gln–Ile–( )–Ser^{9}–Thr^{10}–Pro–Gln–Asp–(Ser)^{15}–Asp–Thr–Glu–Thr^{18}–( )^{19}–Asp^{20}–(Ser)–Asn–(Thr or Leu)–Asp····

Fig. 4. Sequence of the Amino-Terminal Region of Dolabellanin A
( ) means ambiguous identification.

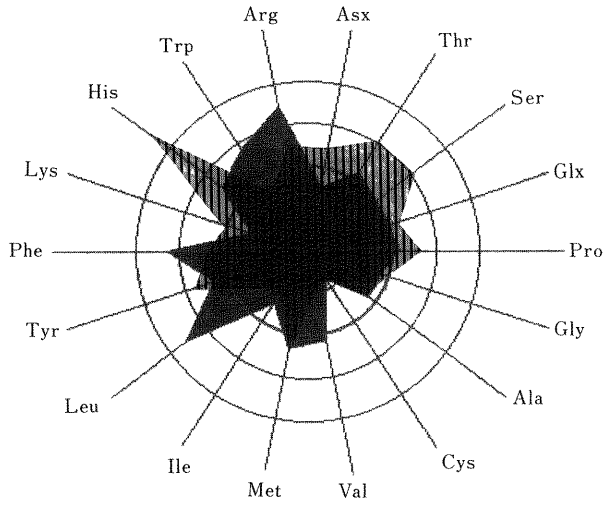

Fig. 5. Comparison of Amino Acid Compositions of Dolabellanin A and Dolabellanin P
Dolabellanin A (■), dolabellanin P (▥).

Discussion

The Indian and Pacific Ocean sea hare, *D. auricularia*, has been reported to contain cytotoxic substances of low molecular weight[1-3]; however, cytotoxic substances of high molecular weight have not previously been reported.

In this work we describe the purification of a novel cytolytic factor (dolabellanin A) from the albumen gland to a homogeneous species of approximately 250 kDa, as judged by gradient gel electrophoresis and gel filtration. The activity was recovered from gel slices in the same position as the main band of protein. This factor appeared to be a glycoprotein with 4 subunits of 70 kDa. Dolabellanin A is distinct from aplysianins previously isolated.[4-7]

We have also isolated a nonspecific cytolytic factor, dolabellanin P, from the purple fluid of *D. auricularia*. Dolabellanin P is a glycoprotein of 60 kDa and is a single polypeptide.[13] However, dolabellanin P does not resemble dolabellanin A in terms of amino acid composition (Fig. 5), kinetics of cytolysis or antitumor activity

TABLE IV. Comparison of Properties of Cytolytic Glycoproteins from *D. auricularia*

Origin	Molecular weight (kDa)	Subunit	Sugar content (%)	Amino acid composition	Cytolysis completion (h)	Cytolysis at 15 °C	Target specificity
Purple fluid	60	1	0.8	Asx, Glx, Ser; rich	2	Positive	Nonspecific
Albumen gland	250	4	10.9	Leu, Glx, Asx; rich	18	Negative	Highly cytotoxic for tumor cells

spectrum (Table IV). Although the characteristics of dolabellanin A will be reported in detail in a later paper, noteworthy characteristics are the high tumor lytic activity even at low concentration (10 ng protein/ml) and the low cytolytic activity on normal cells. These facts suggest that dolabellanin A is a different molecule from dolabellanin P and that sea hares of the *Dolabella* species contain two different cytolytic factors.

The albumen gland contains a large quantity of dolabellanin A (about 10—20% of the total protein), but its physiological function is unknown. We recently found that an egg extract of *Dolabella* species showed cytolytic activity and that the activity of the eggs decreased during hatching (unpublished data). Dolabellanin A therefore may be a precursor of the cytolytic factor of the eggs. Moreover, we found that dolabellanin A has strong antibacterial activity.[14] These findings suggest that this substance may play a defensive role against infection and may regulate hatching and/or embryogenesis of sea hares.

In a following paper we shall report on the characteristics of dolabellanin A, and the mechanisms of its cytolytic action and antitumor activity *in vivo*.

Acknowledgments The authors thank Dr. S. Kikuchi of Chiba University, Dr. I. Hayashi of the University of Tokyo, Dr. S. Nemoto of Ochanomizu University, and Drs. T. Furugori and T. Suzuki of Kochi University for collecting *D. auricularia*. We also thank Dr. Anzai of Nippon University for fruitful discussions. This study was supported in part by a grant from Kotobuki Seiyaku Co., Ltd.

References

1) G. R. Pettit, R. H. Ode, C. L. Herald, R. B. von Dreele, and C. Michel, *J. Am. Chem. Soc.*, **98**, 4677 (1976).
2) C. Ireland, D. J. Faulkner, J. Finer, and J. Clardy, *J. Am. Chem. Soc.*, **98**, 4664 (1976).
3) G. R. Pettit, Y. Kamano, C. L. Herald, A. A. Tuinman, F. E. Boettner, H. Kizu, J. M. Schmidt, L. Baczynskyj, K. B. Tomer, and R. J. Bontems, *J. Am. Chem. Soc.*, **109**, 6883 (1987).
4) J. Kisugi, H. Kamiya, and M. Yamazaki, *Cancer Res.*, **47**, 5649 (1987).
5) H. Kamiya, K. Muramoto, and M. Yamazaki, *Experientia* **42**, 1065 (1986).
6) M. Yamazaki, K. Kimura, J. Kisugi, and H. Kamiya, *FEBS Lett.*, **198**, 25 (1986).
7) H. Kamiya, K. Muramoto, R. Goto, and M. Yamazaki, *Nippon Suisan Gakkaishi*, **54**, 773 (1988).
8) U. K. Laemmli, *Nature* (London), **227**, 680 (1970).
9) J.-Y. Chang, R. Knecht, and D. G. Braun, *Biochem. J.*, **199**, 547 (1981).
10) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
11) K. Muramoto, R. Goto, and H. Kamiya, *Anal. Biochem.*, **162**, 435 (1987).
12) K. Nakamura, and T. Furukohri, *Seikagaku*, **60**, 1060 (1988).
13) M. Yamazaki, S. Tansho, J. Kisugi, K. Muramoto, and H. Kamiya, *Chem. Pharm. Bull.*, **37**, 2179 (1989).
14) M. Yamazaki, J. Kisugi, H. Ohye, Y. Ishii, and H. Kamiya, *Igaku-No-Ayumi*, **146**, 917 (1988).