Purification of a cytolytic factor from purple fluid of a sea hare

Masatoshi Yamazaki, Katsuya Kimura, Jun Kisugi and Hisao Kamiya*

Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Tsukui-gun, Kanagawa 199-01 and **School of Fisheries Sciences, Kitasato University, Sanriku, Iwate 022-01, Japan*

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A cytolytic factor inducing tumor lysis was purified approx. 1000-fold from purple fluid of the sea hare *Aplysia kurodai*. The purified factor appeared homogeneous on polyacrylamide gel electrophoresis and consisted of a single polypeptide chain of 60 kDa. The cytolytic factor was half-maximally active at 3–25 ng protein/ml and lysed all tumor cells tested, but not normal cells.

Cytolytic factor *Tumor lysis* *(Sea hare,* Aplysia*)* *Purple fluid*

1. INTRODUCTION

Sea hares, which belong to the subclass Opisthobranchia of the mollusca, appear to have few predators. When disturbed by enemies, many discharge a purple fluid from the purple gland. The nature of the purple dye in the purple fluid of sea hares was elucidated by Rüdiger [1], but no bioactive substance has yet been detected in this fluid. Here we report the isolation and purification of a cytolytic factor (aplysianin-P) from the purple fluid of *Aplysia kurodai*.

2. MATERIALS AND METHODS

2.1. *Collection of purple fluid of* A. kurodai

Specimens of *A. kurodai* were collected in Lake Hamana, Shizuoka, Japan, in May and June. The purple fluid was obtained by agitating the animals and frozen at −80°C until use.

2.2. *Purification procedure*

The purple fluid was dialyzed against 10 mM phosphate buffer and loaded onto columns (2.9 × 53 cm, 4 columns in parallel) of DE 52 (Whatman, Maidstone) previously equilibrated with starting buffer (10 mM phosphate, pH 7.4). The columns were washed with the starting buffer and then material was eluted with a gradient of 0.2–1 M NaCl. Fractions with cytolytic activity were pooled, concentrated on a Toyo UK-10 membrane and applied to a column (2.7 × 120 cm) of Sepharose 6B. Fractions with activity eluted with phosphate-buffered saline were pooled, concentrated as before and applied to a column (1.9 × 105 cm) of Sephacryl S-200. Fractions with cytolytic activity were dialyzed against 10 mM phosphate buffer and loaded onto a column (1.0 × 1.3 cm) of DE 52 previously equilibrated with 10 mM phosphate buffer, pH 7.4. The cytolytic factor was eluted with a linear gradient of 0–0.4 M NaCl.

2.3. *Tumor cells*

MM46 and MH134 tumor cells were grown in the peritoneal cavity of C3H/He mice, L1210 and P388 leukemic cells in the abdomen of DBA/2 male mice, and EL 4 lymphoma cells in the peritoneal cavity of C57Bl/6 mice. Human leukemic K562 cells, and murine YAC-1 cells were cultured in vitro.

2.4. *Cytolytic assay*

Target cells (2×10^6 cells/ml) were labeled with

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$Na_2{}^{51}CrO_4$ (100 μCi/ml) in RPMI medium containing 10% fetal calf serum for 2 h and then washed 3 times. Labeled target cells (5×10^3 cells) with or without the factor in wells (7 mm diameter) of flat-bottomed microplates were incubated in 0.2 ml of medium 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:

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

2.5. *Protein determination*

Protein was measured by the procedure of Lowry et al. [2] using bovine serum albumin as a standard.

2.6. *Polyacrylamide gel electrophoresis*

SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli [3] 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.

3. RESULTS

A cytolytic factor (aplysianin-P) was purified from the purple fluid (830 ml) by two types of gel filtration and two cycles of ion-exchange chromatography. The elution profiles on column chromatographies are shown in fig.1. The cytolytic activity was eluted in a fraction of about 50 kDa with 260 mM NaCl. The purple dye was completely removed, mainly during the first DE 52 column chromatography and then partly during Sepharose 6B column chromatography. The purification of aplysianin-P is summarized in table 1. The purified colorless preparation gave a single protein band of 60 kDa on SDS-polyacrylamide gel electrophoresis

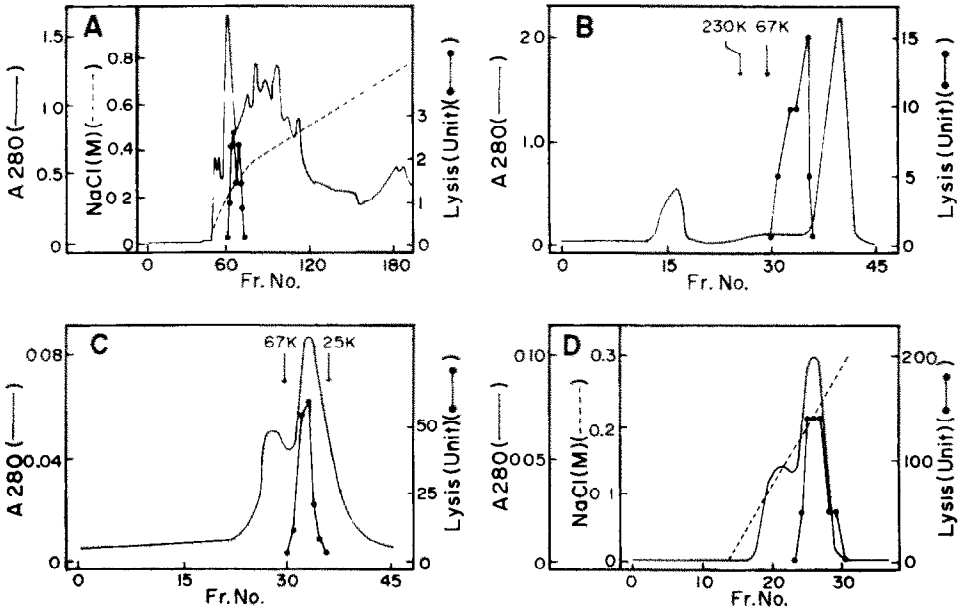

Fig.1. Elution patterns of the cytolytic factor on column chromatographies. (A) DE 52, (B) Sepharose 6B, (C) Sephacryl S-200, (D) DE 52.

Table 1

Summary of purification of aplysianin-P

	Activity (units)	Protein (mg)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
1. Crude fluid	747	2947	0.25	1	100
2. DE 52	329	53	6.2	25	44
3. Sepharose 6B	256	2.6	98.5	394	34
4. Sephacryl S-200	304	1.1	276	1104	41
5. DE 52	156	0.5[a]	306	1224	21

[a] Protein was determined from the absorbance at 280 nm

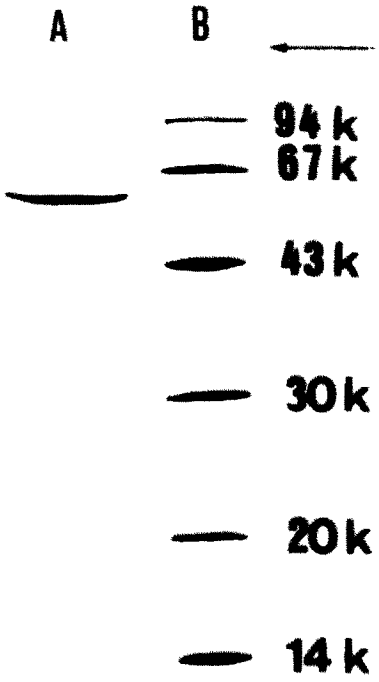

Fig.2. SDS-polyacrylamide gel electrophoresis of the purified factor. (A) The purified sample (20 μg protein) was subjected to electrophoresis on 12.5% acrylamide slab gels at a constant voltage of 120 V for 3 h. (B) The markers used were phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa).

Table 2

Target specificity of aplysianin-P

Target cells	ED_{50} (ng/ml)[a]
MM46 (mammary carcinoma)	10
MH134 (hepatoma)	8
L1210 (leukemia)	3
P388 (leukemia)	5
YAC-1 (lymphoma)	11
EL-4 (lymphoma)	4
K-562 (leukemia)	25
Spleen cells (normal)	>40000
Sheep erythrocytes	>40000

[a] Concentration for 50% lysis of target cells

(fig.2). Treatment of the factor with 2-mercaptoethanol did not affect the molecular mass of the 60 kDa component.

As shown in table 2, the purified factor at 3–25 ng protein/ml caused lysis of all murine and human tumors tested. In contrast, at concentrations up to 40 μg protein/ml it did not affect normal spleen cells or sheep red blood cells. Thus tumor cells were much more susceptible than normal cells to the cytolytic factor in the purple fluid of *A. kurodai*.

4. DISCUSSION

We purified this cytolytic factor (aplysianin-P) from the purple fluid to a homogeneous species of 60 kDa. This factor is distinct from the purple dye previously reported, and is the first bioactive substance found in the purple fluid of a sea hare.

Recently, we reported that eggs of a sea hare

contain an antineoplastic factor (aplysianin-E) that inhibits tumor development in vivo and lyses tumor cells directly in vitro [4]. Aplysianin-E is a glycoprotein of 250 kDa consisting of 3 different subunits [5]. Aplysianin-E and -P have similar activities, but appear to differ in molecular size and subunit structure. Moreover, an antibody against aplysianin-E from sea hare eggs did not react with aplysianin-P from the purple fluid (not shown). It is not clear at present whether aplysianin-P in the purple fluid plays a defensive role, but it is noteworthy that it had high cytolytic activity on tumor cells, even at low concentration (5 ng protein/ml; 1×10^{-10} M), but no cytolytic activity on normal cells.

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