Purification of antineoplastic factor from eggs of a sea hare

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An antineoplastic factor inducing tumor lysis was purified to apparent homogeneity from the supernatant of a homogenate of eggs of the sea hare *Aplysia kurodai* On gel filtration, the purified substance gave a single band of material of 250 kDa containing 3 different subunits and this band coincided with cytolytic activity The purified factor was half-maximally active at 10–60 ng protein/ml and lysed all tumor cells tested, but not normal cells

Antineoplastic factor *Tumor lysis* *Sea hare* Aplysia *Cytolysis*

1. INTRODUCTION

Sea hares of the species *Aplysia* have been reported to contain some biological active substances, including antibacterial factors [1,2], toxins [3,4] and chemical defensive substances [5]. Most of these substances are low-M_r materials derived from seaweeds. However, no bioactive high-M_r substances except agglutinin have previously been identified in sea hares [6].

Recently, we found an antineoplastic factor in eggs of *Aplysia kurodai* [7]. Here we report the purification of this factor and its identification as high-molecular-mass substances of 250 kDa.

2. MATERIALS AND METHODS

2.1. *Collection of eggs of* A. kurodai

Eggs of *A. kurodai* were collected from Lake Hamana, Shizuoka, Japan, in May and June, and were frozen at −80°C until use.

2.2. *Extraction of antineoplastic factor*

Egg masses of *A. kurodai* were homogenized with 2 vols of 0.85% saline for 10 min, and the homogenate was centrifuged at 10000 rpm for 30 min. The supernatant was recentrifuged at 40000 rpm for 60 min to obtain a clear supernatant.

2.3. *Tumor cells*

MM46 and MM48 tumor cells were collected from the peritoneal cavity of C3H/He mice. L1210 leukemic cells were collected from ascites of DBA/2 male mice. EL 4 cells were obtained from the peritoneal cavity of C57BL/6 mice. Human leukemic cells, Raji, Molt-3, and K-562, were harvested from in vitro cultures. Cells of a human lung adenocarcinoma line, PC-6, were also obtained from in vitro cultures These human tumor cells were kindly supplied from Drs N. Saijo and A Shimizu (National Cancer Center, Tokyo).

2.4. *Cytolytic assay*

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. 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 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:

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

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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 and carbohydrate determination*

Protein was measured by the procedure of Lowry et al [8] using bovine serum albumin as a standard. Carbohydrate was determined by the method of Dubois et al [9] using glucose as a standard

3. RESULTS

An antineoplastic factor was purified from an egg homogenate by two types of gel filtration, ion-exchange chromatography and then chromatofocusing The cytolytic activity was eluted in fractions of about 230 kDa (fig.1) Fractions with activity were concentrated on an ultrafiltration membrane (Toyo Kagaku, Tokyo) and applied to a column (1 8 × 106 cm) of Sephacryl S-300 (Pharmacia, NJ) with phosphate buffer. Fractions with cytolytic activity were dialyzed against 10 mM phosphate buffer and loaded onto column (0.6 × 8.5 cm) of DE 52 (Whatman, Maidstone) previously equilibrated with the starting buffer (10 mM phosphate, pH 7 4). The column was washed with the starting buffer and then material

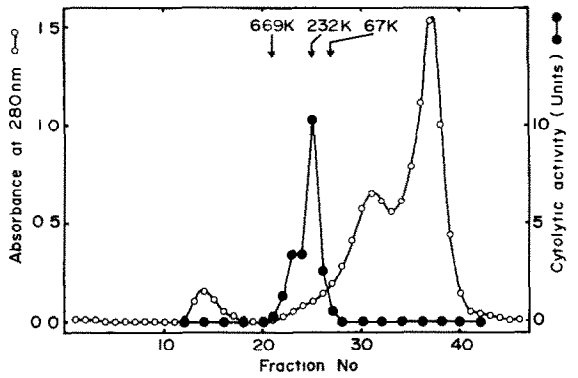

Fig 1 Gel filtration of antineoplastic factor from eggs of a sea hare The supernatant a homogenate of the eggs was applied to a Sepharose 6B column (2 6 × 118 cm) and fractions of eluate were examined for cytolytic activity (●) and absorbance at 280 nm (○) Bovine serum albumin (67 kDa), catalase (232 kDa) and thyroglobulin (669 kDa) were used as marker proteins

was eluted with a linear NaCl gradient (0–200 mM). Table 1 summarises the purification. The specific activity was not increased by the step of chromatofocusing (pH 7.3). The purified factor at a concentration of 30 ng protein/ml lysed murine MM46 tumor cells.

To confirm that the purified factor was associated with cytolytic activity, we subjected the purified factor to electrophoresis on 4–30% acrylamide gel and sliced the gradient gel to assay the cytolytic activity of the slices. As shown in fig 2, the purified sample gave a single band of

Table 1

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Step	Activity (units)	Protein (mg)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Homogenate[a]	13500	1180	11	1	100
Sepharose 6B	6000	78	77	7	44
Sephacryl S-300	5550	35	158	14	41
DEAE-cellulose	2170	6 7	323	29	16
Chromatofocusing[b]	2210	6 3	352	32	16

[a] Eggs (120 g) were homogenized with 250 ml of 0 85% saline The homogenate was centrifuged to obtain a clear supernatant as described in section 2

[b] Samples were not pooled at this step Thus total activity was calculated as the sum of the activities of each fraction on chromatofocusing

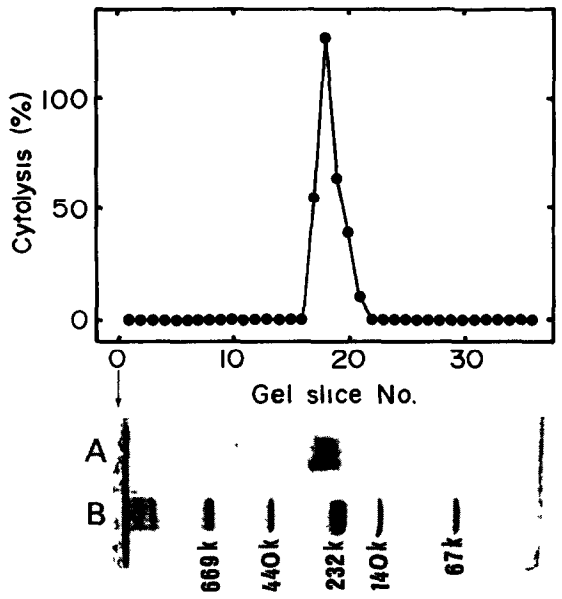

Fig 2 Gradient gel electrophoresis of purified factor The purified factor was subjected to electrophoresis on 4–30% acrylamide slab gel at a constant voltage of 125 V for 15 h (A) Purified factor (30 μg protein) (B) Molecular mass markers (thyroglobulin 669 kDa, ferritin 440 kDa, catalase 232 kDa, lactate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

material of 250 kDa and the position of cytolytic activity coincided with that of the band To examine the subunits of the cytolytic factor, we subjected the purified sample to SDS-polyacrylamide gel electrophoresis. Fig 3 shows that the purified preparation gave 3 main bands of 76, 88 and 102 kDa, respectively. Treatment of the factor with 2-mercaptoethanol did not affect the M_r of these 3 components.

As shown in fig 4, all the murine and human tumors tested were lysed completely by the purified factor at concentrations of 10–60 ng protein/ml. In contrast, normal spleen cells and red blood cells were resistant to this cytolytic factor at concentrations up to 2 μg protein/ml. These results indicate that cells are relatively susceptible to the cytolytic factor from the eggs of *A. kurodai*

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) Sample of homogenate (115 μg protein) (C) Purified sample (21 μg protein)

4. DISCUSSION

Recently, we reported that the egg mass of *A. kurodai* contains an antineoplastic factor that inhibits tumor development in vivo and lyses tumor cells directly in vitro [7].

Here we purified this antineoplastic factor to a homogeneous species of approx. 250 kDa, as judged by gradient gel electrophoresis The activity was recovered from gel slices in the same position as the single band of protein. This factor seemed to be composed of 3 distinct subunits, but it is still

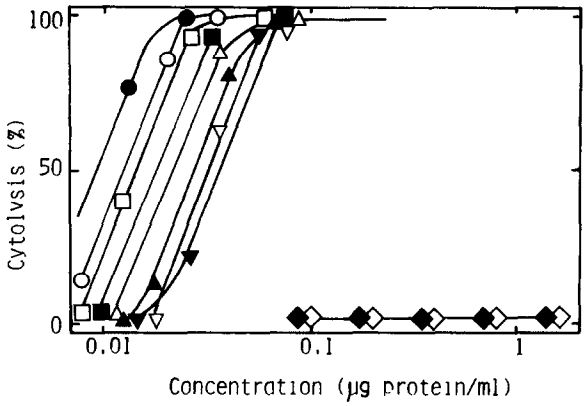

Fig 4. Cytolytic activity of purified sample Target cells were incubated with the purified factor for 18 h (▽) MM46, (■) MM48, (○) L1210, (△) EL4, (●) Raji, (□) Molt-3, (▲) K562, (▼) PC-6, (◇) murine spleen cells, (◆) sheep red blood cells

uncertain whether each of these subunits has cytolytic activity on tumor cells. Since the purified sample contained 8% sugar, the antineoplastic factor seems to be a glycoprotein with 3 subunits.

It is noteworthy that even at low concentrations (10 ng protein/ml; 4×10^{-11} M; 30000-fold dilution of extract) this factor had high cytolytic activity on tumor cells, but low cytolytic activity on normal cells. The latter characteristic is consistent with the low toxicity of this factor in vivo [7]. These features suggest that this factor may be a useful substance.

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