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Full Length Research Paper

Isolation of a 60 kDa protein with *in vitro* anticancer activity against human cancer cell lines from the purple fluid of the Persian Gulf sea hare, *Aplysia dactylomela*

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Sea hares have greatly attracted the interest of all those investigating chemical defense substances. Most of these substances are low molecular weight compounds derived from algal diets. *In vitro* anticancer effect of a 60 kDa protein isolated from the purple fluid of *Aplysia dactylomela* on four human cancer cell lines was investigated in this study. A 60 kDa protein was purified from secreted purple fluid of *A. dactylomela*, a sea hare from Persian Gulf. The protein purification procedure consisted basically of ammonium sulfate precipitation, ion exchange chromatography using DEAE–Sepharose and ultra-filtration method. *In vitro* antiproliferative and cytotoxic activity of the protein of interest were evaluated on L929, K562, HL60 and NB4 human cancer cell lines. The antiproliferative and cytotxic effects of 60 kDa protein on human cancer cell lines were measured by MTT assay. Results showed that the 60 kDa protein of the purple fluid of *A. dactylomela* exhibited the antiproliferative effect on human cancer cell lines, especially on NB4 cell line. It was maximally active at 0.5 – 1.5 µg/ml on NB4 cell line. Interestingly, the protein did not show significant cytotoxic effects.

Key words: Sea hare, anticancer, neoplastic, *Aplysia*.

INTRODUCTION

Nowadays, cancer is one of the most lethal diseases in human beings. Therefore, investigations for finding new anticancer compounds are imperative and interesting. After taking into consideration the immense side effects of synthetic anticancer drugs, many researchers are making concerted efforts to find new and natural anticancer compounds. Therefore, marine organisms, because of their accessibility and their therapeutic applications for many other diseases, are of interest. In some studies on marine organisms, identification and isolation of some compounds with anticancer and antitumor activity were reported (Alcaro et al., 2003; Amador et al., 2003; Kamiya et al., 1998; Kisugi et al., 1987; Takamatsu et al., 1995; Wessels et al., 2000; Yamazaki, 1989).

Sea hares are opistobranch molluscs which have attracted the interest of biologists investigating biologically active compounds (Falkner et al., 1973; Kinnel et al., 1977; Yamamura and Terada, 1977). *Aplysia* is one of the sea hares genus and in the present research *Aplysia dactylomela* from the Persian Gulf was studied. A 250 kDa glycoprotein named *Aplysia*nin E was purified from the egg of *Aplysia kurodai* which exhibited anticancer activity against some marine and human tumor cell lines as well as on experimentally tumoral mice (Kisugi et al., 1987). In another study, a 320 kDa glycolprotein was isolated from the albumen gland of *A. kurodai* with anti-tumor activity (Takamatsu et al., 1995). Dolastatin 10 is another anticancer protein which was isolated from *Dolabella auricularia*, a sea hare originating from the Indian Ocean (Turner et al., 1998).

Most *Aplysia* species discharge a purple fluid from a

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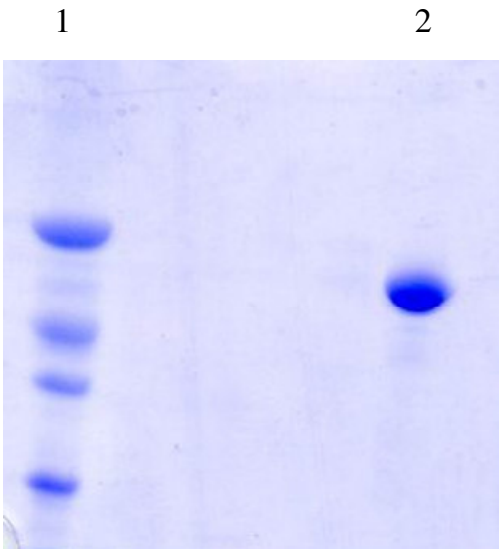

Figure 1. Result of protein purification in SDS-PAGE. **Lane 1.** Protein size marker (66 kDa, 45 kDa, 36 kDa, 24 kDa). **Lane 2.** Purified 60 kDa proteins from purple fluid of *A. dactylomela*.

purple gland when they are disturbed and in some studies, the presence of a 60 kDa protein with different effects such as, antibacterial activity, hemagglutination activity and antitumor activity in purple fluid of sea hares were reported (Kamiya et al., 1989; Yamazaki et al., 1989). On the other hand there is no research on the anticancer activity of 60 kDa protein of purple fluid of the species *A. dactylomela*. Therefore, in this study, isolation and purification of the 60 kDa protein from the purple fluid of *A. dactylomela* from Bushehr port in Iran was done and its *in vitro* anticancer activity on some human cancer cell lines was measured.

MATERIALS AND METHODS

Sea hare

A. dactylomela species was taken from the west coast of Bushehr port, south west Iran. The purple fluid was obtained by agitating the sea hares and after collection was kept at -80°C until use.

Cell lines

In this study, three suspension human cancer cell lines, K562, NB4, HL60 and an adherent monolayer human cancer cell line, L929 were used. These were obtained from the hematology department of Tarbiat Modarres University.

Cell culture

K562, NB4 and HL60 cells were cultured in RPMI 1640 (Gibco) cell culture medium containing 10% fetal bovine serum (Gibco). L929 cells were grown in DMEM (GIBCO) cell culture medium containing 10% fetal bovine serum. All cultured cell lines were incubated at 37°C in the presence of 5% CO_2 (Morgan and Darling, 1992).

Cell viability test

The trypan blue exclusion test was used for detection of cell viability (Morgan and Darling, 1992).

MTT assay test

10^4 viable cells were cultured in a 96-well cell culture microplate (NUNC) by using DMEM cell culture medium containing 10% fetal bovine serum. Then, each concentration of 60 kDa protein were added to the wells of microplate and then incubated at 37°C in the presence of 5% CO_2 for 48 h. For each concentration of protein, three wells were chosen as negative control by adding PBS only. After 48 h post treatment of cells with different concentrations of 60 kDa protein, 0.1 mL of 5 mg/ml MTT solution (Sigma) were added to each well of microplate. The Microplate was incubated at 37°C for 4 h and then, the optical density of each well was read by the ELISA reader in 540 nm (Van de Loosdrecht et al., 1994).

Protein purification

By using 85% ammonium sulfate solution, all proteins in purple fluid of *A. dactylomela* were precipitated (Copeland, 1994). The precipitated proteins were loaded on DEAE- Sepharose column (pharmacia), which was equilibrated by 20 mM Tris buffer, pH 7.4. Then the column was washed by 0 - 1 M NaCl gradient and the fractions at the bottom of the column were collected and tested with spectrophotometer at 280 nm for protein determination. Fractions with acceptable ODs were dialyzed against 20 mM Tris buffer, pH 7.4. Further purification was done by using an ultra filter with 30 kDa pore size (Amicon) (Scopes, 1994).

SDS-PAGE analysis

Protein samples were separated by SDS-PAGE under denaturing conditions in 12.5% polyacrylamide gels (Laemelli, 1970).

Protein concentration assay

Protein concentration determined by the Bradford method.

RESULTS

Protein concentration determination which was done by the Bradford method showed that the amount of proteins in purple fluid of *A. dactylomela* was 260 µg/ml and its quantity after precipitation by 85% ammonium sulfate raised to 330 µg/ml. Based on the result from SDS-PAGE on purified purple fluid (Figure 1) the presence of a band with 60 kDa molecular weight was established. That band showed the protein of interest.

Antiproliferative activity of different amounts of precipitated crude proteins and purified 60 kDa protein against cancer cell lines were measured by OD values resulting from MTT assay test (Table 1). As shown in Table 1, purified 60 kDa protein showed an acceptable inhibitory effect on cancer cell lines especially NB4 and L929 cell

Table 1. Results of MTT assay (OD values in 540 nm) in different concentration of precipitated crude proteins and purified 60 kDa proteins on four types of cancer cell lines.

Type of Protein	Concentration (µg/ml)	Type of Cell Line			
		L929	NB4	K562	HL60
Precipitated crude Proteins	0.0	0.704	0.698	0.701	0.702
	0.5	0.623	0.552	0.645	0.610
	1.0	0.615	0.536	0.600	0.602
	1.5	0.598	0.504	0.592	0.599
Purified 60 kDa Proteins	0.0	0.698	0.702	0.704	0.695
	0.5	0.598	0.505	0.585	0.540
	1.0	0.520	0.435	0.571	0.530
	1.5	0.510	0.320	0.520	0.523

Table 2. Results of cytotoxicity of precipitated crude proteins and purified 60 kDa protein on NB4 cell line.

Sample	Viable cell number	Viable cell percentage
PBS (Negative control)	6×10^4	97%
Precipitated crude proteins	4×10^4	88%
Purified 60 kDa protein	1×10^4	86%

lines. The best inhibitory effect on cell proliferation was for the NB4 cells.

The purified 60 kDa protein was more effective than precipitated crude proteins. For example, the effect of 1.5 µg/ml of precipitated crude proteins was equal to the effect of 0.5 µg/ml of purified 60 kDa protein when they were tested on NB4 and L929 cell lines. To determine the cytotoxicity of 60 kDa protein and precipitated crude proteins the viability of treated and control negative cells were determined (Table 2). As shown in Table 2, the percentage of NB4 cells 48 h post treatment by 0.5 µg/ml of purified 60 kDa protein was 86%. The same test for precipitated crude proteins was done and the percentage of viable cells was 88%. The data in Table 2 shows that the number of viable NB4 cells, 48 h post treatment by 0.5 µg/ml of purified 60 kDa protein, did not increase compared to the number of cells (10^4 cell) which were inoculated to each well of cell culture micro-plate at first. However, the number of viable cells in the negative control wells increased from 10^4 to 6×10^4 cells at the same time. Also in samples which were treated by precipitated crude proteins the number of viable cells increased from 10^4 to 4×10^4 cells.

DISCUSSION

In this study *A. dactylomela* was searched for in the Jofreh coast of Busher port. Although in the other reports this species of sea hare originating from Brazil and Spain were studied for the effects of some its natural metabolites (Melo et al., 2000). In a study on *A. dactylomela*, three kinds of natural compounds were identified which had cytotoxic effects on some cancer cell lines such as MCF7, HMO2 and HEPG4. In that research, the cytotoxic concentration of isolated anticancer metabolites was 1 to 7 µg/ml (Wessels et al., 2000). Meanwhile the 60 kDa protein, which was isolated from the purple fluid of *A. dactylomela* in Brazil and tested for its antibacterial and hemagglutination activities, was named dactylomelin P (Melo et al., 2000).

A 60 kDa protein was isolated from the secreted purple fluid of *A. kurodai* and called Aplysianin P. Aplysianin P displayed cytolytic and antibacterial effects. Also, the effect of Aplysianin P as a nucleic acid synthesis inhibitor was established (Yamazaki et al., 1989). 1 µg/ml of the purified 60 kDa showed antiproliferative activity against all tested cell lines but the significant effect was on NB4 cell line. In another research, which was done on Aplysianin P of *A. kurodai*, the anticancer effect of that protein was determined at 3 to 25 µg/ml for different cancer cell lines (Yamazaki et al., 1989). Interestingly, based on data from Table 2, the purified 60 kDa did not exhibit cytotoxic effects on NB4 cells. However, in another study, the cytolytic effect of its counterpart from *A. kurodai* was reported (Yamazaki et al., 1990). Such differences could be caused and changed by many elements such as type of cell line, species of sea hare or other unidentified elements. In this study the 60 kDa protein was purified from the secreted purple fluid of *A. dactylomela* by using ion exchange chromatography and ultrafiltration methods. Meanwhile, other protein purification methods such as gel filtration and specific ion exchange chromatography were used in some studies

(Yamazaki et al., 1989). Regarding the final 60 kDa purified protein concentration which was 170 µg/ml compared to the total protein concentration in crude purple fluid which was 260 µg/ml, methods utilized for protein purification in our study were fairly appropriate. Some studies applied radioactive materials for anti-proliferative or cytotoxicity tests; but in this study, MTT assay was done which does not require any radioactive material for its accuracy (Kamiya et al., 1986; Kisugi et al., 1987).

Regarding the antiproliferative effect of purified 60 kDa in this study and the reported severe cytolytic effect of Aplysianin P from *A. kurodai*, further investigations on other properties of 60 kDa protein and the mechanism of antiproliferative action(s) are necessary. In conclusion, the purified 60 kDa protein of secreted purple fluid of *A. dactylomela* from Persian Gulf has anti-proliferative effect on some human cancer cell lines. Based on the results of this research the most effective anti-proliferative activity of 60 kDa protein was on the NB4 cell line without any apparent cytotoxic effects. Therefore, more studies on other cancer cell lines and *in vivo* antitumor evaluation of that protein are recommended.

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