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Cytotoxicity and L-Amino Acid Oxidase Activity of Animal Venoms

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The cytotoxicity of animal venoms (snakes, insects and marine animals) was measured against SNU-1 (stomach cancer cells) by dye uptake assay (MTT method). And also L-amino acid oxidase (AAO) activity of the venoms was compared. Among them, the venom from *Ophiophagus hannah* (king cobra) showed a strong AAO activity as well as a high potent cytotoxicity. Cytotoxic protein having a AAO was then partially purified by HPLC-GPC and two fractions (Fr. I and Fr. II) were collected. The IC_{50} values of Fr. I and Fr. II were 0.19 μg/ml and 1.36 μg/ml, respectively. The results suggested that the cytotoxicity of king cobra venom may be due to its AAO activity.

Key words : Venoms, Cytotoxic protein, L-Amino acid oxidase

INTRODUCTION

Venoms and toxins of natural origin have unique pharmacological properties whether they are obtained from plants, microorganisms, or animals (Tu, 1991; Harvey, 1995). On account of this reason toxins can also be used scientifically, or as the good lead compounds in the design of new therapeutic agents. Animal venoms from numerous land snakes, insects and marine animals have been shown to possess cytotoxic or lytic effects on tumor cells *in vitro* (Newman *et al.*, 1993). Therefore, such venom compounds may be potential anticancer agents when used in combination with other anticancer drugs.

The components of snake venoms that are cytotoxic to cancer cells are phospholipase A_2 (PLA_2) (Newman *et al.*, 1993), phospholipase C (PLC) (Markland, 1990), cytotoxins (Dufton and Hider 1988; Chiou *et al.*, 1993), and L-amino acid oxidase (AAO) (Stocker, 1990). Of them PLA_2, PLC, and cytotoxins showed direct lytic effects on cells, while in vitro cytotoxic mechanism of AAO may be due to the generation of hydrogen peroxide (Terada *et al.*, 1988). But there have been few reports showing the relationship of cytotoxicity with the level of AAO so far. Here we presented cytotoxicity and AAO actvity of twenty nine animal venoms to demonstrate a relationship. Of them king cobra venom (*Ophiophagus hannah*) showed a very low IC_{50} value against stomach cancer cells, so a partial purification of the enzyme was performed.

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MATERIALS AND METHODS

Materials

The venoms were purchased from Sigma Chemical Co. (St. Louis, USA). TSK- G3000SW (0.75×30 cm) was purchased from Tosoh Corporation (Japan). Mini-PROTEAN®II electrophoresis cell was products of Bio-Rad (Richmond, USA). Other reagents were from Sigma Chemical Co (USA).

Cell lines

The cell line, SNU-1 (stomach cancer cell line: CRL 597.1) established by College of Medicine, Cancer Research Institute, Seoul National University, Korea, was maintained under monolayer conditions in RPMI 1640 medium (GIBCO, New York, USA) supplemented with 10% newborn calf serum (GIBCO), 1 mmol/L L-glutamine, 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate (Sigma) at 37°C in a humidified atmosphere of 5% CO_2 in air.

Measurement of cytotoxicity

Cytotoxicity of animal venoms was tested against SNU-1 cell line by MTT {3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide} assay according to the procedures as described previously (Carmichael *et al.*, 1987; Scudiero *et al.*, 1988). In brief, rapidly

growing cells were harvested, counted, and inoculated at the appropriate concentrations (180 μl volume) into 96-well microtiter plates using a multichannel pipet. After cells were exposed to the test venoms (20 μl) for 4 days at 37°C, 50 μl of MTT stock solution (5 mg/ml in PBS) was added to each well and the plate was incubated for 4 h. After aspiration of the medium, DMSO (150 μl) was added to solubilize the MTT-formazan product. After 30 min at room temperature, the plate was read on a microplate reader on a 540 nm. The IC_{50} (50% inhibitory concentration; μg/ml) was defined as the amount of cytotoxin causing a inhibition of 50% in the viability of the cells compared with the control cell culture.

Partial purification of cytotoxic protein from king cobra venom

Crude venom of *Ophiophagus hannah* (1 mg) was applied to TSK G3000SW-HPLC equilibrated with 40 mM Tris-HCl (pH 7.4) containing 0.1 M NaCl at a flow rate of 0.5 ml/min and the eluant was monitored at 280 nm. Two major fractions (Fr. I and Fr. II) were collected and the cytotoxicity was measured. Fr. I showing a cytotoxic activity was dialyzed against distilled water. The dialyzed solution was concentrated and its purity was compared on SDS-PAGE.

L-Amino acid oxidase assay

L-Amino acid oxidase activity was determined spectrophotometrically with L-leucine as a substrate as described previously (Holme and Goldberg, 1975). UV spectrophotometer (JASCO, MHT-344, Japan) was equipped with thermal cuvetts by an external circulating water bath. The principle of the reaction is to determine the amount of ammonia liberated from the substrates by L-amino acid oxidases, which may be coupled with NADH-dependent reductive amination of 2-ketoglutarate catalysed by an exogeneous dehydrogenase. In brief, the mixture was composed of 30 mM 2-ketoglutarate (0.2 ml), 2.25 mM NADH (0.2 ml), 15 mM ADP (0.1 ml), 22.5 U glutamate dehydrogenase (Boerhringer Mannheim, Germany), venoms (1 mg/ml, 0.2 ml), and 0.1 M L-leucine (0.1 ml) in 0.1 M glycylglycine buffer (pH 7.5) and was incubated at 37°C for 10 min. Then the resulting fall in absorbance at 340 nm could be calculated as an enzyme activity. One unit of activity was defined as the amount of oxidative deamination of 1.0 mole L-leucine per min at 37°C.

Protein assay

Protein was measured using Coomassie blue G250 according to the Bradford method (Bradford, 1976). Bovine serum albumin was used as standard.

RESULTS

The data for cytotoxicity and amino acid oxidase activity were described in Table I. Of the twenty nine animal venoms, twenty two were originated from snake and the rest of them were from marines and insects. The IC_{50} values of eight venoms showed a concentration lower than 3.0 μg/ml against SNU-1 cells (Table I). The venoms showing a low IC_{50} values are from *Agkistrodon piscivorus piscivorus, Crotalus atrox, Crotalus ruber, Crotalus adamanteus, Naja nigricollis crawshawii, Naja nigricollis nigricollis* and *Ophiophagus hannah*. We have previously reported a fibrinolytic enzyme from *Agkistrodon piscivorus piscivorus* (Hahn *et al.*, 1995). When we compared the results of cytotoxicity with AAO activity, some correlations could be seen. Especially *Ophiophagus hannah* (king cobra) venom showed a high AAO activity as well as a potent cytotoxicity.

The venom of king cobra snake was partially purified by HPLC-GPC (Fig. 1). Two major peaks were collected and their cytotoxities were measured (Table

Table I. Cytotoxicity and L-amino acid oxidase activity of animal venoms against SNU-1

Scientific name	IC_{50} (μg/ml)	AAO activity (U/mg venom)
Agkistrodon contortrix mokasen	7.2	0.15
Agkistrodon piscivorus piscivorus	<3.0	7.28
Bitis gabonia	220.0	5.11
Bothrops atrox	3.5	0.63
Bungarus multicinctus	44.5	3.69
Bitis arientans	5.0	5.11
Crotalus atrox	<3.0	1.98
Crotalus horridus horridus	6.3	trace
Crotalus ruber	<3.0	1.01
Crotalus adamanteus	<3.0	0.39
Dendroaspis angusticeps	21.7	trace
Echis carinatus	<3.0	3.57
Naja nigricollis crawshawii	<3.0	1.28
Naja nigricollis nigricollis	<3.0	0.75
Naja nivea	12.0	0.42
Naja melanoleuca	11.5	1.93
Naja haje	4.0	0.73
Naja naja kaouthia	6.2	1.93
*Ophiophagus hannah**	<3.0	20.58
Pseudechis porphyriacus	12.3	1.25
Sepedon hemachatus	5.0	9.12
Viper russelli	300.0	0.56
Diadema antillarum sea urchin	6.0	0.24
Condylactis gigantea anemone	8.3	0.48
Scorpio maurus palmatus scorpin	39.1	0.97
Chrysaora quinquecirrha jellyfish	3.1	2.45
Heloderma horridum lizard	3.1	0.63
Apis mellifera bee	3.1	trace
Actinopyga agassizi trepang	58.1	trace

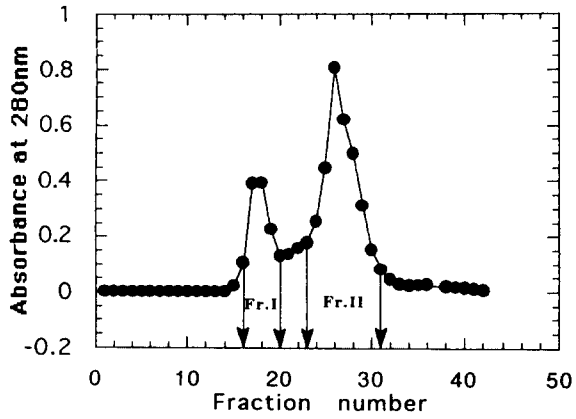

Fig. 1. Purification of cytotoxic protein from king cobra venom by HPLC-GPC. Purification was performed by TSK G3000SW column and two fractions were collected. Cytotoxicity of each fraction was assayed.

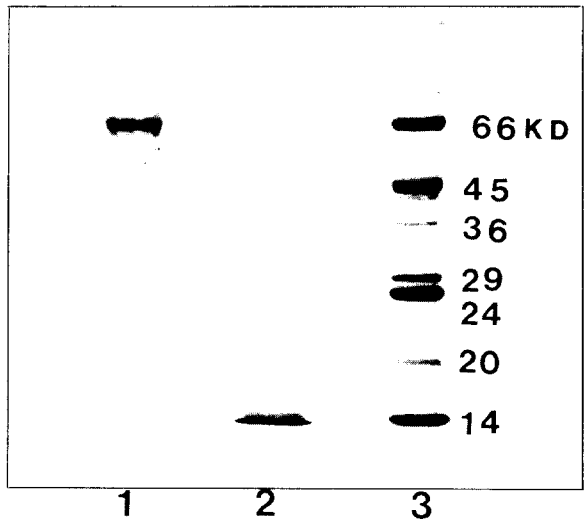

Fig. 2. SDS-PAGE (10%) of Fr I and Fr II from TSK G3000SW-HPLC. Lane 1, Fr I; Lane 2, Fr II; Lane 3, Molecular markers

II). The first peak, Fr. I, showed a strong inhibition (IC_{50}=0.19 μg/ml) against stomach cancer cells. Its molecular weight was almost 75,000 Da under the reducing conditions of SDS gel. Under the native conditions the molecular weight of AAO was found to be 150,000 Da. A full description of purification will be described elsewhere (Ahn, *et al.*). The second peak is in the range of 15,000 Da. And a strong activity of AAO was seen in Fr. I (data not shown). The results strongly suggest AAO and cytotoxicity may have some correlations.

DISCUSSION

While microbial- and plant-derived products have traditionally been thought of as sources of novel anticancer components, toxins of animal origin have received considerably less attention. But the recent discoveries of marine products such as bryostatin and dolastin-10 representing the inhibitors of PKC and tubulin, respectivey, suggest that other animal toxins or components derived from animals may be useful as anticancer agents (Newman *et al.*, 1993).

Table II. Cytotoxicity of TSK fractions of *Ophiophagus hannah*

Fraction	IC_{50}* (μg/ml)
Crude venom	<3.0
Fr 1	0.19
Fr 2	1.36

*Cytotoxicity against SNU-1 cell line by MTT assay

Some trials have been done to prevent the growth of tumor cells using a fibrinolytic enzyme from *Crotalus adamanteus*. It is likely to prevent tumor cell from adhering to host and metastasizing by lysis of platelet aggregation and attack of natural killer cell (Markland, 1990). Also, venoms of Elapid snakes, in particular, those of *Naja nigricollis*, are much more potent for lysing tumor cells than the venoms of Viperids and Crotalids (Chaim-Matyas and Ovadia, 1987).

Many authors have reported individual variability of rattlesnake venoms (Tan and Ponnudurai, 1991). Particulary northworthy is the individual variation in the cytotoxicity and variability of L-amino acid oxidase activity of many animal venoms. In this experiment, the correlation of king cobra venom between L-amino acid oxidase activity and cytotoxicity can be found, but other venoms showing low IC_{50} values (<3.0 μg/ml) should be further studied in terms of cytotoxic components, for instance, phospholipase A_2, cytotoxins, fibrinolytic enzymes or other toxins. In fact, there are some reports showing their cytotoxicities: *Agkistrodon piscivorus piscivorus* (fibrinoloytic and L-amino acid oxidase activity, Nikai *et al.*, 1988), *Crotalus atrox* (fibrinolytic activity, Tan and Ponnudurai, 1991), *Crotalus adamanteus* (L-amino acid oxidase activity, Tan and Ponnudurai, 1991), *Crotalus ruber* (phospholipase A_2 activity, Tan and Ponnudurai, 1991), *Naja nigricollis nigricollis* (cytotoxin P4, Chaim-Matyas *et al.*, 1991) and *Echis carinatus* (carinatin: a potent inhibitor of platelet aggregation, Stocker, 1990).

This result tells us AAO may be one of the cytotoxic proteins and should be further characterized for the mechanism.

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