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Inhibitory effects on phospholipase A2 and antivenin activity of melanin extracted from *Thea sinensis* Linn

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Abstract

Antivenin activity of melanin extracted from black tea (MEBT) was reported for the first time. The antagonistic effect of MEBT was evaluated for *Agkistrodon contortrix laticinctu*s (broadbanded copperhead), *Agkistrodon halys blomhoffii* (Japanese mamushi), and *Crotalus atrox* (western diamondback rattlesnake) snake venoms administered i.p. to ICR mice. MEBT was injected i.p. immediately after the venom administration in dose of 3 mg per mouse in the same place of venom injection. MEBT demonstrated neutralization effect against all venoms tested. The greatest antivenin effect of MEBT was found against Japanese mamushi snake venom. In this case, half the mice died within 2.5 ± 0.7 h after injection of 0.9 mg/kg of venom. An immediate injection of MEBT substantially reduced the toxic effect of venom and extended time at the 50% level of survival up to 52.3 ± 2.3 h. The antivenin activity of MEBT is due to chelating of Ca^{++} and non-specific binding of phospholipase A2. The inhibitory effect of MEBT on phospholipase A2 assessed for different venoms was similar to that obtained with pure enzyme. Low toxicity of MEBT in combination with its antagonistic activity against different venoms may allow effective life-saving treatment against snakebites. Such application of MEBT is important when identification of the snake is impossible or if specific treatment is unavailable.

Keywords: Melanin extracted from black tea; Phospholipase A2; Inhibitory effect; Antivenin activity

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Introduction

The infusion of tea leaves was known in ancient China as a detoxifying medicine (Balentine et al., 1997). The modern data about the properties of tea are mainly associated with properties of its low molecular polyphenols, but scarce information concerning the polymeric compounds of tea is available. Recently, we have reported (Sava et al., 2001a) a biopolymer of melanin natural in tea (*Thea sinensis* Linn.). Similar melanin pigments extracted from various sources were intensively studied earlier (Nicolaus, 1968; Prota, 1998), and the most significant properties concerning melanin chelating and free radical properties were reported. Melanin extracted from black tea (MEBT) represents the high molecular part of tea polyphenols (Sava et al., 2001b) with physico-chemical characteristics that are similar to those of typical melanin. However, MEBT revealed unexpected protective activity against various toxic substances (Sava et al., 2002; Sava et al., 2003; Hung et al., 2003).

Recently, we have reported the antagonistic effect of MEBT against snake venoms. Development of antivenin agents is in an active research area and is mostly concentrated on the application of specific anti-serums. Each anti-venom serum works effectively as a specific neutralizing agent for the designated snake venom (Heard et al., 1999). However, certain specific antisera are not always available at the time of the snakebite. Also, an unambiguous identification of the snake is not always available. Therefore, a universal neutralizing agent, which could be used for immediate life-saving treatment for all kinds of snakebites, is needed.

Toxicity of snake venom is often addressed (Dennis et al., 1981) to the enzymatic activities of phospholipase A_2 (PLA_2, EC 3.1.1.4). PLA_2 is a major component of snake venom specifically catalyzing the hydrolysis of fatty acid ester bonds at position 2 of 1.2-diacyl-sn-3phosphoglycerides. In addition to the digestion of phosphoglycerides, PLA_2 exhibits wide varieties of pharmacological effects such as neurotoxicity, cardiotoxicity, myotoxicity, necrosis, anticoagulation, hypotensivity, hemolysis, haemorrhage, and edema (Kini, 1997). The interaction of melanin with enzymes was recently demonstrated (Eckhart et al., 2000). It was evidenced that melanin is able to bind reversibly to thermostable DNA polymerase and inhibit its activity. However, the melanin effect on PLA_2 is not known.

Among non-specific antivenins, a variety of substances has been studied using PLA_2 (Odell et al., 1998; Kocholaty, 1966; Lindahl and Tagesson, 1997). In particular, the inhibitory effect of some polyphenols on venom PLA_2 obtained from *Vipera russelli* and *Crotalus atrox* snakes was investigated (Lindahl and Tagesson, 1997). It was found that polyphenols could inhibit PLA_2 and may be used for antivenin treatment. However, biotransformation of polyphenols reduces the efficacy of their inhibitory effect.

MEBT represents the unhydrolyzed complex of tea polyphenols (Sava et al., 2001a), which can be considered as a prospective antivenin agent. The present work focused on the study of the effect of MEBT on different snake venoms and aimed to evaluate the possibility of its application for the first-aid treatment against various snake venoms to provide the elimination or delaying of a lethal toxic effect.

Materials and methods

Materials

In our experiments we used fully fermented Chinese black tea (*Thea sinensis* Linn.). The black tea was purchased in local retail shops (Taichung, Taiwan). Mr. Nien-Yung Chiu has identified the teas in

the Institute of Chinese Pharmaceutical Sciences, China Medical College, and a voucher specimen (GSH-001) is deposited in the Herbarium of this Institute. Snake venom PLA_2 was purchased from Worthington Biochemical Corporation (Freehold, NJ). *Agkistrodon contortrix laticinctu*s (broadbanded copperhead), *Agkistrodon halys blomhoffii* (Japanese mamushi), and *Crotalus atrox* (western diamond-back rattlesnake) snake venoms were purchased from Sigma Chemicals Co. (St. Louis, MO). Synthetic melanin also was purchased from Sigma Chemical Co. and was used as a reference compound without any further treatment. All additional reagents were of chemical reagent grade and purchased from Merck KGaA (Darmstadt, Germany).

Isolation and characterization of MEBT

Isolation of MEBT was performed according to previously reported procedure (Sava et al., 2001a) employed with minor adjustments. The extraction time was limited to 12 h to avoid excessive oxidation of MEBT. After extraction, the mixture was filtered and centrifuged at 15,000 g for 30 min to obtain MEBT extract. This extract was acidified by the addition of 2N HCl to pH 2.5 and centrifuged at 15,000 g for 15 min to pellet form. Acid hydrolysis was employed for purification of MEBT. The purified product thus obtained was dissolved in 0.2% NH_4OH, and the solution was subjected to repeated precipitations. Four precipitations were employed to sequester MEBT from low molecular impurity and to improve its homogeneity.

MEBT was further purified on a Sephadex G-75 column and the fractions were monitored by UV absorbance at 280 nm. To evaluate the molecular mass (MM) of MEBT, the column was calibrated with bovine serum albumin (MM 66,000), carbonic anhydrase (MM 29,000), cytochrome C (MM 12,400), and aprotinin (MM 6,500) as size markers.

Physical and chemical characterizations of MEBT were performed according to conventional procedures (Nicolaus, 1968; Prota, 1998). Ultraviolet-visible (UV) absorption spectra were obtained with a JASCO V-530 UV-Visible Spectrophotometer (Jasco Ltd, Great Dunmow, UK). Infrared (IR) spectra were recorded for KBr samples on a Perkin-Elmer spectrometer 1600 FT (Perkin-Elmer Instruments, Norwalk, CT). Standard tests for melanin were performed. Tests include solubility in water, aqueous acid, and common organic solvents; oxidative bleaching using $KMnO_4$, $K_2Cr_2O_7$, NaOCl, and H_2O_2; and positive reaction for polyphenols.

MEBT solution was prepared by the following procedure. The purified preparation was dissolved in distilled water slightly alkalized by 0.5N NH_4OH to pH 9, incubated for 1 h at 50 °C, followed by centrifugation at 10,000 × g for 15 min. The ammonia was removed by a rotary evaporator under reduced pressure to a final pH of 7.5. Final concentration of MEBT was adjusted to 30 mg/ml. The solution was filtered through Nalgene 0.45 μm syringe filter and then autoclaved for 1 h at 125 °C.

Evaluation of MEBT toxicity (LD_{50})

Experiments were performed on healthy adult ICR mice with body weight of 24 ± 1 g. All animal experiments were conducted in accordance with the Institutional Guide for the Care and Use of Laboratory Animals and performed under the supervision of the Animal Center at China Medical College. Animals were housed on a standard rodent chow and water ad libitum. Seventy-two mice of

either sex were equally distributed into 9 groups with an equal number of both sexes in each group. The first group of untreated animals was considered as a control, and the other 8 groups were treated with 30 mg/ml aqueous solution of MEBT given intraperitoneally in doses of 500–1200 mg/kg of body weight. The animals were investigated throughout an observation period of 72 h, and medium lethal dose (LD_{50}) values were calculated by means of probit analysis according to Weber (1980). Significance of the differences between groups was considered at $P < 0.05$ using Student's t-test.

In vivo assessment of venom toxicity and anti-venom effect of MEBT

*Agkistrodon contortrix laticinctu*s (broadbanded copperhead), *Agkistrodon halys blomhoffii* (Japanese mamushi), and *Crotalus atrox* (western diamondback rattlesnake) snake venoms were used. All venom solutions were prepared in physiological saline, stored at 4 °C and warmed up to 37 °C before injection. LD_{50} was determined as described above.

For the in vivo evaluation for the antivenin effect of MEBT, 16 animals were equally divided into two groups. All animals received i.p. doses of 3-fold LD_{50} of venom. The experimental group received 3 mg MEBT per mouse immediately after the venom administration in the same place of venom injection. The lethality of mice was monitored for 48 h, and an effective time (ET_{50}) representing 50% level of animal survival was determined from 3 replications of experiment.

In vitro assesment of MEBT inhibitory effect

Effect of MEBT on PLA_2 was evaluated using neutralization of venom by MEBT. A mixture containing 20 μl of venom and 20 μl of MEBT was incubated on ice, and after 10 min of incubation a 10 μl sample was withdrawn for PLA_2 activity assay. Before assessment of activities, all venoms and MEBT were incubated in various concentrations, and mixtures were analyzed using SDS-PAGE. Namely, after incubation with MEBT, snake venoms were centrifuged at 10,000 rpm for 3 minutes, and 10 ìl of supernatants were boiled for 3 min with 2% (v/v) 2-mercaptoethanol and loaded onto an SDS/l0% polyacrylamide gel. Separations were carried out at a constant 200 V for 50 min using Mini-Protean II apparatus (BioRad). Gels were stained with Coomassie Brilliant blue R-250. The intensities of major protein bands of PLA_2 were quantified with densitometer and plotted against concentration MEBT. The purified PLA_2 enzyme was used in concentration 0.5 mg/ml. Snake venoms were employed in concentrations of 5 mg/ml for *Agkistrodon contortrix laticinctu*s and *Agkistrodon halys blomhoffii*, and of 10 mg/ml for *Crotalus atrox.* MEBT was taken at a concentration of 10 mg/ml.

PLA_2 activity was determined using assay based on release of free fatty acids from L-phosphatidyl-choline (Evans and Ownby, 1999) employed with minimal adjustments. Accumulation of free fatty acids was monitored at 578 nm using cresol red as indicator. L-phosphatidylcholine solution was prepared by dissolving 1 g of L-phosphatidylcholine and 3 ml Triton X-100 in 10 ml of methanol and bringing solution to 20 ml with distilled water. The indicator solution was prepared by combining 1.5 μg cresol red and 250 μl methanol and bringing solution to 25 ml with distilled water. Solutions of 0.1 M glycylglycine and 90 mM calcium chloride were prepared with distilled water. L-phosphatidylcholine solution (4 ml), indicator solution (7 ml), glycylglycine solution (3 ml), and calcium chloride solution (1 ml) were mixed to obtain buffered substrate solution (BSS). The pH of BSS was adjusted to 9.5 using

1.0 N NaOH, and volume was brought to 30 ml with distilled water. For the evaluation of PLA_2 activity, 1 ml of BSS was placed into spectrophotometric cuvette, and blank absorbance was recorded. The absorbance was recorded again in 1 min after the addition of 10 μl of venom sample to the cuvette, PLA_2 activity (**P**) was determined by the formula:

$$\mathbf{P}(\text{Unit/mg}) = \frac{101 \times \mathbf{A}}{\mathbf{E} \times \mathbf{C}}$$

where **A** was a change in optical absorbance measured during 1 min after venom addition; **E** was an extinction coefficient determined as a change in absorbance during 1 min after addition of 10 μl of 0.1 N HCl to 1 ml of BSS, and **C** was a venom sample concentration.

Statistical analysis

All data were expressed as mean ± S.E.M. Differences between groups were considered to be significant at $P < 0.05$ using Student's t-test.

Results and Discussion

MEBT was extracted from black tea as previously reported (Sava et al., 2001a) with minor adjustments. In particular, extraction time was diminished to 12 h to avoid excessive oxidation of MEBT. The average yield of MEBT obtained after purification was 1.9%. Final separation of MEBT using Sephadex G-75 yielded one major (92%) and one minor fraction with molecular masses of 14 ± 3 kDa and 8 ± 3 kDa, respectively. Further study of MLP was carried out on the major fraction.

The purified preparation of MEBT exhibited all the physical and chemical properties common to natural melanin previously reported (Nicolaus, 1968; Prota, 1998; Ellis and Griffiths, 1974; Bilinska, 1996; Paim et al., 1990). It was insoluble in organic solvents (ethanol, hexane, acetone, benzene, and chloroform); dissolved only in alkali; precipitated below pH 3 and in alkaline FeCl3; was bleached by H2O2, KMnO4, K2Cr2O7, and NaOCl; and produced a blue color with FeSO4/ferricyanide. The solution of MEBT in 0.1 M phosphate buffer (pH 8.0) exhibited strong optical absorbance similar to synthetic melanin. IR-spectroscopy of MEBT demonstrated similar structural peculiarities compared to previously studied melanin pigments including synthetic melanin (Bilinska, 1996; Paim et al., 1990) and allowed research into the interaction between MEBT and Ca^{++} ions. The IR spectrum of MEBT showed a broad band at 3450 cm-1, attributed to stretching vibrations of -OH and -NH2 groups. A strong absorption at 1650 cm-1 was recognized as the vibrations of aromatic C = C or C = O groups. After the acid hydrolysis of MEBT the intensity of both bands at 3450 cm-1 and 1650 cm-1 was reduced, a phenomenon caused by the reaction between phenolic and carboxylic groups owing to the formation of lactones (Sava et al., 2001a). The strong absorbance at 1720 cm-1 was assigned to stretching vibrations of free carboxylic groups. Interaction with Ca^{++} decreased this band, but acid treatment of MEBT restored its intensity. Such behavior may have been caused by chelating properties of MEBT, which likely are involved in various biological effects.

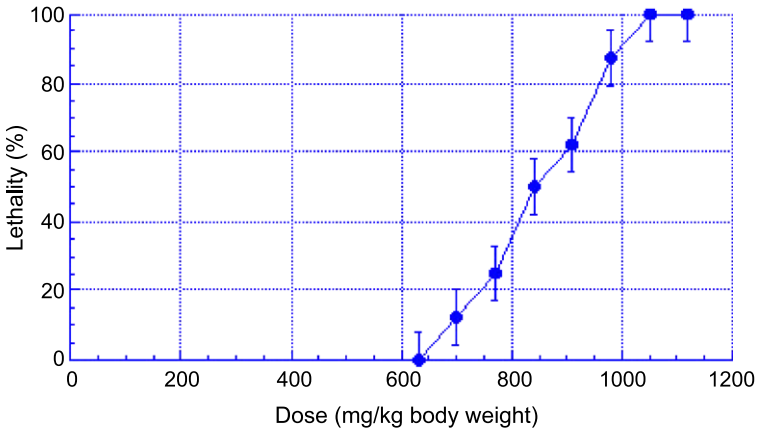

Fig. 1. Dose-response curve obtained for intraperitoneal administration of MEBT to ICR mice. Deaths were recorded within 72 h of observation and lethality was calculated for each group containing 8 animals.

The acute toxicity of MEBT was estimated from the results represented in Fig. 1. The median lethal dose (LD_{50}) for IRC mice injected i.p. with MEBT was 868 mg/kg. No statistical difference in the acute toxicity was found between male and female animals. Administration of MEBT to animal in higher doses from 770 to 910 mg/kg demonstrated mild stimulation of central nervous system. Highest doses up to 1050 mg/kg caused a noticeable acceleration of respiration and tremor. Administration of doses higher then 1050 mg/kg led to manifestation of convulsion. However, no toxic effects were reported below doses of 560 mg/kg of body weight. Thus, toxicity of MEBT can be qualified as low or very low.

For evaluation of acute toxicity of snake venoms, ICR mice were injected i.p. in doses 0.1–1.9 mg/kg body weight. The highest toxicity was found for the broadbanded copperhead snake venom. For this venom, LD_{50} was 0.2 mg/kg of body weight. The Japenese mamushi and rattlesnake venoms were less toxic demonstrating LD_{50} values of 0.3 and 0.6 mg/kg, respectively.

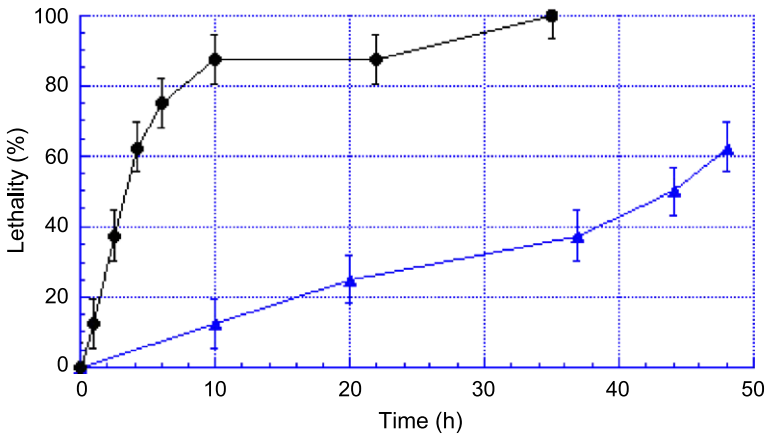

Fig. 2. Lethality of ICR mice receiving i.p. injection of rattlesnake venom in dose 1.7 mg/kg alone (circles) and additional i.p. injection of MEBT in dose 3 mg per mouse (triangles).

Table 1
Effect of MEBT on survival of ICR mice injected with different snake venoms

Snake venoms	Dose of venom injected (mg/kg)	Effective time of survival, ET_{50} (h)*	
		Venom alone	Venom + MEBT**
Rattlesnake venom	1.7	4.2 ± 0.7	44.1 ± 1.3
Broadbanded copperhead snake venom	0.6	5.3 ± 1.3	45.5 ± 2.5
Japanise mamushi venom	0.9	2.5 ± 0.7	52.3 ± 2.3

*The values of ET_{50} represent time required for 50% of animal survival. The results expressed as mean ± SD of 3 replications.

**MEBT was injected i.p. in dose 3 mg per mouse immediately after injection of venom.

The antagonistic effect of MEBT against snakebite was evaluated by i.p. venom injection at dose 3-fold higher of LD_{50}. MEBT was injected i.p. immediately after the venom administration in dose of 3 mg per mouse approximately in the same place of venom injection. The effective time (ET_{50}) of 50% survival of mice was employed for the evaluation of MEBT effect against venom toxicity. As shown in Fig. 2, the peritoneal injection 1.7 mg/kg of rattlesnake venom killed half the mice within 4.2 hours. Further injection of 3 mg of MEBT prolonged the 50% survival to 44.1 hours (Fig. 2). The incubation venom in mixture with MEBT 1 h prior to injection caused additional reduction of venomous toxicity (data not shown). However, no significant difference between the separate injections and injection of mixture was found.

The other venoms from *Agkistrodon contortrix laticinctus* (broadbanded copperhead snake) and *Agkistrodon halys blomhoffii* (Japanese mamushi snake) were tested for investigation of specificity of MEBT. For broadbanded copperhead snake venom, whose acute toxicity was three times higher compared to the venom from rattkesnake (LD_{50} was 0.2 mg/kg), the 50% level of suvival was achieved in 5.3 ± 1.3 h (Table 1). Administration of the same dose of MEBT increased ET_{50} value to 45.5 ± 2.5 h. The greatest antivenin effect of MEBT was found against Japanese mamushi snake venom (LD_{50} was 0.3 mg/kg). In this case, half the mice died within 2.5 ± 0.7 h after injection of 0.9 mg/kg of venom. An immediate injection of 3 mg of MEBT per mouse substantially reduced the toxic effect of venom and extended time at the 50% level of survival up to 52.3 h., 21 times more than that obtained for administration of venom alone (Table 1).

Toxicity of snake venom is often addressed (Dennis et al., 1981) to the enzymatic activities of PLA_2 as a major component of snake venom. PLA_2 from various snake venoms differs widely in its spectra of

Table 2
In vitro inhibitory effect of MEBT towards PLA_2 of various snake venoms compared to purified enzyme

Experimental conditions	PLA_2 activities in different venoms (U/mg)			Activity of purified PLA_2 (U/mg)
	Venom of rattlesnake	Venom of broadbanded copperhead snake	Venom of Japanese mamushi snake	
Venom alone	117 ± 9.3 (100)*	79.7 ± 5.5 (100)	123.6 ± 8.4 (100)	54.7 ± 2.6 (100)
Venom after incubation with MEBT**	78.1 ± 6,5 (66.5)	52.7 ± 2.8 (66.1)	77.8 ± 3.7 (62.9)	31.3 ± 1.1 (57.1)

*Data in parentheses represent the relative activities of PLA_2 as compared with respective activity of venom employed alone. The ctivity values represent the average of three independent experiments.

**The mixture was incubated on ice for 10 min.

toxin actions, although PLA_2 shares a high degree of homology in amino acid sequence and enzyme active sites (Heinrikson, 1991; Scott and Sigler, 1994).

Effect of MEBT on PLA_2 activity was evaluated in vitro. The results obtained on purified PLA_2 enzyme and venoms from rattlesnake (Crota los atrox), broadbanded copperhead (*Agkistrodon contortrix laticinctus*) and Japanese mamushi (*Agkistrodon halys blomhoffii*) are shown in Table 2. Incubation of MEBT caused 43% decrease in specific activity of PLA_2. For other venoms, a reduction from 34% to 37% of PLA_2 activity in presence of MEBT was observed (Table 2). Thus MEBT inhibited PLA2 activities of snake venoms in a manner similar to pure enzyme.

The inhibitory effect of MEBT could be explained by chelating Ca^{++}, an important co-factor of PLA_2 (Dennis, 1981). This possibility was reported from IR-spectroscopic data described above. However, it was difficult to quantify this effect. The inhibition on PLA_2 activity of snake venom might due to direct binding of MEBT. To investigate this possibility, we have incubated PLA_2 with MEBT and precipitated MEBT-PLA_2 complex under mild acidic conditions. After centrifugation the supernatant containing free

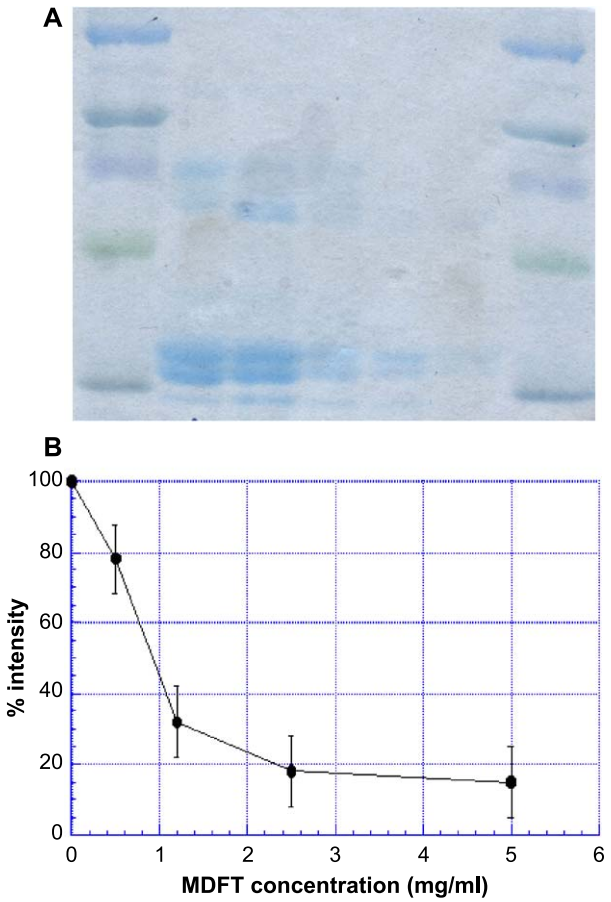

Fig. 3. SDS-PAGE of supernatant obtained after centrifugation of mixture of MEBT and PLA_2 subjected to incubation on ice for 10 min (panel A). Final concentrations of MEBT for each lane were as follows: 0.0 mg/ml (lane 1), 0.5 mg/ml (lane 2), 1.25 mg/ml (lane 3), 2.5 mg/ml (lane 4), and 5.0 mg/ml (lane 5). The intensities of protein bands were quantified, normalized, averaged, and plotted against concentration of MEBT (panel B).

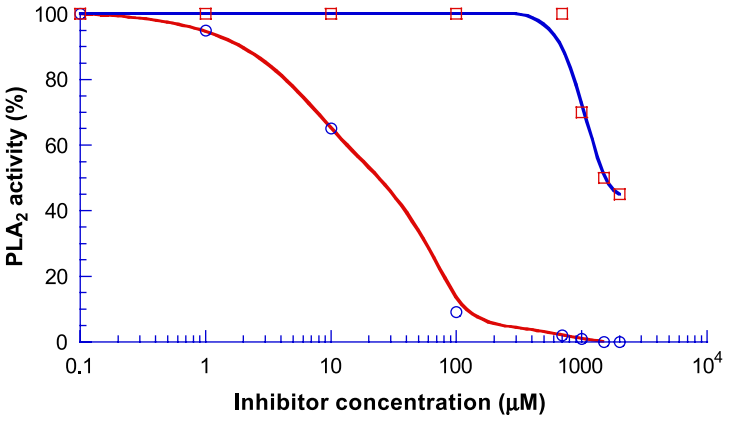

Fig. 4. Dose response curves demonstrated inhibition of PLA by MEBT (squares) compared to specific and competitive inhibition by p-bromophenacyl bromide (circles).

PLA_2 was analyzed by SDS-PAGE (Fig. 3). It was found that MEBT bound all components of PLA_2 in a non-specific manner. Increase of MEBT concentration resulted in proportional decrease of all protein components. Binding activity reached the maximal level at concentration of 4–5 mg/ml MEBT. At this concentration the molar ratio between free and bound PLA_2 was about 1/5.

To further explore the non-specific nature of MEBT inhibition against snake venom, the dose-response curve of MEBT was compared to p-bromophenacyl bromide, a specific and competitive inhibitor for PLA_2 (Fig. 4). Inhibition of PLA by MEBT exhibited steep curve, which differs from inhibition by the specific and competitive inhibitor.

Conclusion

Antivenin activity of MEBT was reported for the first time. There was no documented antivenin activity for the other melanin pigments. These properties of MEBT might be useful for antiveninous therapy. The advantage of MEBT application is based on its low inherent toxicity and un-hydrolyzed polymeric nature. The antivenin activity of MEBT is likely due to chelating of Ca^{++} and binding the protein components of venom. We demonstrated that MEBT is capable of partially inhibiting activity of PLA_2 in venom derived from various snakes. The presented results evidence that MEBT can bind all components presented in venomous protein composition, that is, the non-specific inhibitory affect of MEBT. In addition to PLA_2 snake venoms contain many other toxic enzymes. The possibility of inactivating these enzymes similar to PLA_2 inactivation could be due to the non-specific character of binding.

Low toxicity of MEBT in combination with its antagonistic activity against the different venoms allowed effective life-saving treatment. Such application of MEBT is important when identification of the snake is impossible or if specific treatment is unavailable. Also, the reduction of toxicity may be needed before specific treatment. From this point, non-specific remedies against venomous injury of unknown origin based on MEBT application presents an alternative in addition to the traditional approaches.

We demonstrated correlation between antivenin activity of MEBT and PLA_2 inhibition as a possible explanation for the protective effect. However, such correlation may only serve as a initial approach. Further research is in progress to study effects of MEBT on other enzymes of snake venoms and effects on cytochrome P450. It will be intriguing that different venoms might be inactivated by single compound. The non-specific antivenin activity that matched non-specific binding and inhibition of PLA_2 are subject for further investigation. It is also important to explore the limitation of MEBT protective effect. The variations in dose response for many different snake venoms including sea-snakes, box jellyfish, blue-ringed octopus and stonefish will be of interest for the future research.

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