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Effects of Chinese Snake Venoms on Blood Coagulation, Purified Coagulation Factors and Synthetic Chromogenic Substrates

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Abstract. -We examined the action of venoms from common Chinese *Crotalidae* and *Elapidae* snakes on blood coagulation mechanisms. Procoagulant effects were observed with venoms from *Agkistrodon acutus, Trimeresurus stejnegeri, Ophiophagus hannah* and *Bungarus fasciatus*, the latter two only in the presence of Ca^{2+}. After treatment with a serine protease inhibitor (phenylmethanesulfonyl fluoride, PMSF), *Agkistrodon acutus* venom lost its ability to clot purified fibrinogen but retained its capacity to clot human plasma in the absence of Ca^{2+}. An anticoagulant action was obtained with venoms from *Trimeresurus mucrosquamatus, Agkistrodon halys* and *Naja naja atra.* This action was abolished after treatment with a specific inhibitor of PLA_2 activity (*p*-bromophenacyl bromide, BrPBr), revealing a procoagulant action with high concentrations of treated venoms in the cases of *Trimeresurus mucrosquamatus* and *Agkistrodon halys.* The effects of these venoms on hemostasis have been further characterized by measuring their phospholipase A_2 activity, their ability to hydrolyze synthetic chromogenic substrates and to activate purified blood coagulation factors (prothrombin, factor X, protein C and plasminogen). These venoms showed an amidolytic activity which was mainly due to serine proteases (90 to 95% of inhibition with PMSF). Combining the observations obtained with human plasma and purified blood coagulation factors, we concluded that: i) six of the eight tested Chinese venoms (*i.e.*: *Ophiophagus hannah, Bungarus fasciatus, Agkistrodon acutus, Trimeresurus mucrosquamatus, Trimeresurus stejnegeri* and *Naja naja atra*) contain components which activate factor X in a Ca^{2+}-dependent manner; ii) three venoms (*Agkistrodon acutus, Agkistrodon halys* and *Trimeresurus stejnegeri*) contain prothrombin activators; iii) *Ophiophagus hannah* venom has a weak protein C activating activity; and iv) *Trimeresurus stejnegeri* venom possesses plasminogen activating activity. In addition, several of these venoms have previously been shown to contain thrombin-like and fibrinogenolytic enzymes, anticoagulant phospholipases A_2 (PLA_2s) and/or non enzymatic anticoagulant components.

Key Words: Snake venoms, blood coagulation, purified blood coagulation factors, chromogenic substrates.

Introduction

Snake venoms are known to be a rich source of hydrolytic enzymes, mainly proteases and phospholipases A_2 and of non-enzymatic proteins, which induce disorders of blood coagulation, hemorrhage and shock (Pirkle and Markland, 1988; Ouyang and Teng, 1972; Teng and Seegers, 1981). Many proteases acting on different steps of the blood coagulation cascade have been purified from snake venoms. They cleave blood coagulation factors, either in a specific or in a non-specific manner, and cause acceleration or retardation of blood coagulation (Pirkle and Markland, 1988). Some of these proteases, such as thrombin-like enzymes (Stocker and Meier, 1988) or protein C activators (Kisiel et al., 1987), are serine proteases which may be rapidly and irreversibly inactivated by alkylation with PMSF. Other procoagulant or anticoagulant proteases, like factor X or prothrombin activators from *Bothrops atrox* venom (Hofmann and Bon, 1987a; 1987b) or from *Echis carinatus* venom (Morita and Iwanaga, 1978) are insensitive to PMSF and have been postulated to be metalloenzymes. PLA_2s have also been recognized for their anticoagulant activity, which has been attributed to their ability to antagonize the procoagulant action of negatively charged phospholipids (Ouyang et al., 1978).

In order to better understand the pathophysiological action of snake venoms on haemostasis, and to examine the potential use of their procoagulant or anticoagulant components as pharmacological tools, we examined the

effects of various snake venoms on blood coagulation mechanisms *in vitro*, using human plasma, purified blood coagulation factors (fibrinogen, prothrombin, factor X, protein C and plasminogen), and synthetic chromogenic substrates. We examined in detail venoms from the eight most common venomous snakes in China; four belonging to the *Elapidae* family (*Ophiophagus hannah, Naja naja atra, Bungarus fasciatus* and *Bungarus multicinctus*) and the other four to the *Crotalidae* family (*Trimeresurus mucrosquamatus, Trimeresurus stejnegeri, Agkistrodon halys* and *Agkistrodon acutus*).

Methods

Venoms were supplied by the Kunming Institute of Zoology (Academia Sinica, China). The venoms were collected from snakes living in the southern provinces of China and stored desiccated. They were dissolved in 50 mM Tris-HCl buffer, pH 7.8, at a concentration of 1 mg·ml^{-1} and were used immediately.

Bovine factor X, human prothrombin and human Glu-plasminogen were obtained from Sigma (St. Louis, MO, USA). Human protein C was obtained from Diagnostica Stago (Asnières, France). Human fibrinogen (grade L) from Kabi Vitrum (Stockholm, Sweden) was treated with diisopropylfluorophosphate according to the instructions of the manufacturer, in order to irreversibly inactivate traces of thrombin or other blood coagulation factors. Platelet-poor human plasma was the supernatant of human blood mixed with 1/10 volume of 3.8% sodium citrate and centrifuged at 3000 rpm for 15 min. Pools of normal citrated plasma obtained from 5-10 healthy donors were stored at -20°C.

Chromogenic substrates H-D-Phe-Pip-Arg-pNA (S-2238), H-D-Val-Leu-Lys-pNA (S-2251), H-D-Val-Leu-Arg-pNA (S-2266), H-D-Pro-Phe-Arg-pNA (S-2302) and Bz-Ile-Glu-Gly-Arg-pNA (S-2222) were obtained from Kabi Vitrum (Stockholm, Sweden) and the chromogenic substrate H-D-Lys(Cbo)-Pro-Arg-pNA (CBS65-25) was from Diagnostica Stago (Asnières, France). Phenylmethanesulfonyl fluoride (PMSF) and *p*-bromophenacyl bromide (BrPBr) were purchased from Sigma (St. Louis, MO, USA). All other reagents were of the highest purity available.

Chemical modifications

Inactivation of serine proteases by PMSF was performed in 50 mM Tris-HCl, pH 7.8. Venom samples (2 mg·ml^{-1}) were incubated at 37°C for two hours with 5 mM PMSF (stock solution: 0.1 M in dimethylsulfoxid). Inactivation of PLA_2s was carried out in the same buffer by incubating the venom (1 mg·ml^{-1}) at 37°C for one hour with 2 mM BrPBr (stock solution: 0.1 M in acetone). Treated venom samples were then dialyzed for 4 to 8 hours against large volumes of the same buffer.

Chromogenic assays

Amidolytic activity was measured with a Kontron spectrophotometer in 1 cm path-length plastic cuvettes. Assays were performed in 500 ml of 50 mM Tris-HCl, pH 7.8, containing the appropriate chromogenic substrate (0.2 mM). The reactions was initiated by addition of the sample to be tested (5 mg·ml^{-1} to 100 mg·ml^{-1}, final concentrations) and the formation of *p*-nitroanilide was monitored at 405 nm. The amount of substrate hydrolyzed was calculated using a molar extinction coefficient of 10,000 M^{-1}·cm^{-1} for free *p*-nitroanilide.

Determination of PLA_2 activity

PLA_2 activity was determined by the titrimetric method described by Desnuelle et al. (1955), according to the procedure of Radvanyi and Bon (1982).

Effects of the venoms on blood coagulation

Citrated platelet-poor human plasma (200 ml) was incubated at 37°C for 1 min, then a 20 ml aliquot of diluted venom samples was added and clotting time was recorded. In some cases, 5 ml of $CaCl_2$

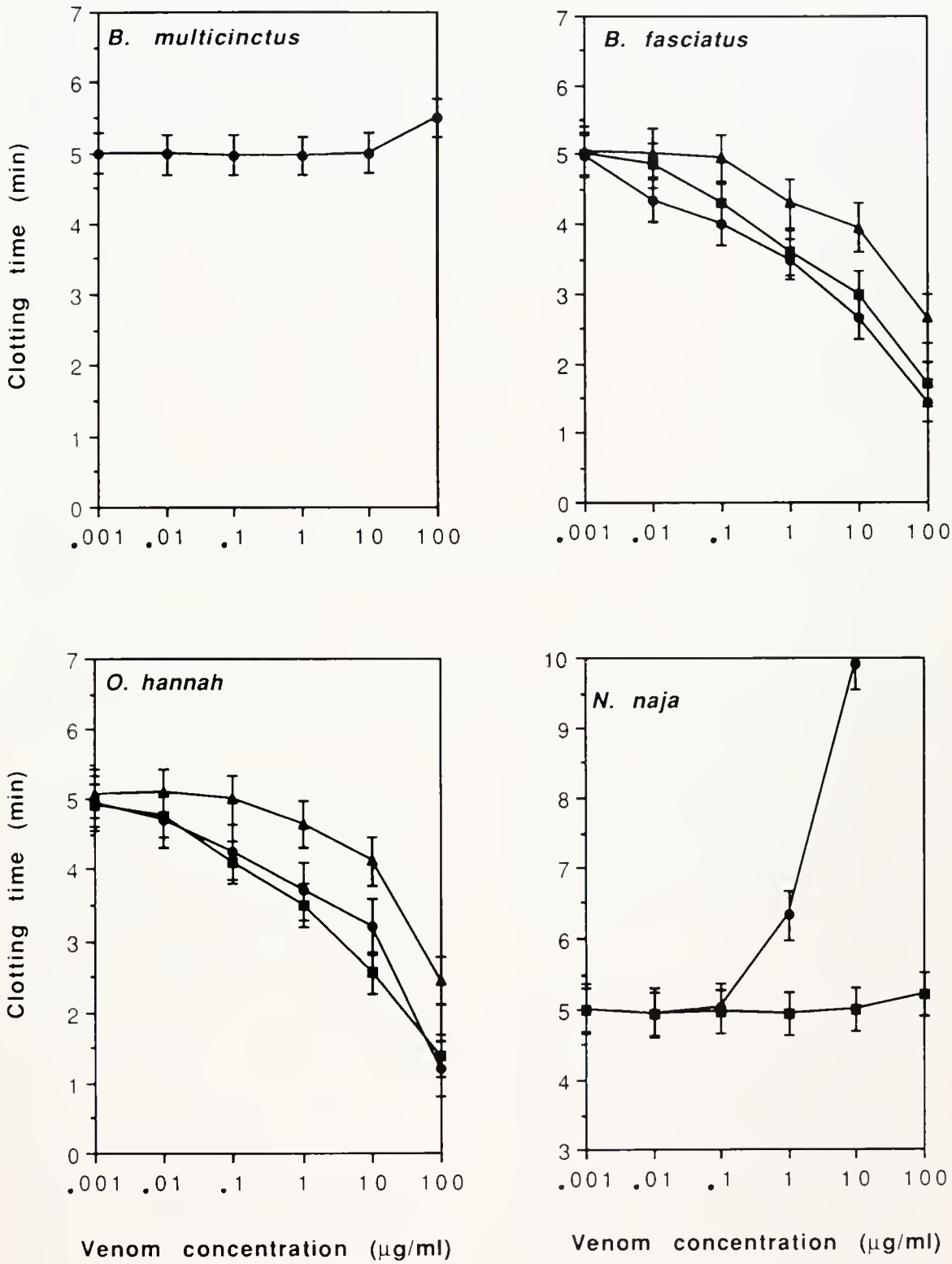

FIGURE 1. Effects of *Elapidae* snake venoms on blood coagulation. Citrated platelet-poor human plasma (200 ml) was incubated at 37°C for 1 min; dilutions of the sample to be tested (20 ml) were then added simultaneously with 5 ml of 0.45 M $CaCl_2$ (10 mM final concentration); native venom (●), PMSF-treated venom (▲) or *p*-bromophenacyl bromide-treated venom (■). Values are the mean of triplicates.

TABLE 1. Phospholipase A_2 activity of the venoms from the common Chinese venomous snakes ($\mu nmol \cdot min^{-1} \cdot mg^{-1}$).

	native venom	treated venoms
Agkistrodon acutus	37±2	<1.0
Agkistrodon halys	220±9	<1.0
Trimeresurus mucrosquamatus	48±3	<1.0
Trimeresurus stejnegeri	50±6	<1.0
Ophiophagus hannah	95±8	<1.0
Bungarus fasciatus	160±11	<1.0
Bungarus multicinctus	180±12	<1.0
Naja naja	35±4	<1.0

The phospholipase A_2 activity of the venoms were determined as described by Radvanyi and Bon (1982) using egg lecithin solubilized by sodium cholate. Venoms were treated by *p*-bromophenacyl bromide as indicated in the method then dialyzed to remove the excess of reagent. Each value is the mean of three determinations ± standard deviation.

(10 mM final concentration) were added simultaneously with the venom samples. Thrombin-like activity was determined by measuring the clotting time of purified human fibrinogen (0.5%) in 50 mM Tris-HCl, pH 7.8, containing 0.1 M NaCl. Fibrinogen (200 ml) was incubated for 2 min at 37°C before addition of 20 ml of diluted venom samples.

Activation of blood coagulation factors by snake venoms

Activation of prothrombin and factor X was performed as described by Hofmann and Bon (1987a; 1987b). Briefly, purified human prothrombin (50 $mg \cdot ml^{-1}$) was incubated at 37°C in 50 mM Tris-HCl, pH 7.8, containing 0.1 M NaCl and different concentrations of the samples to be tested. Aliquots (50 ml) were removed at various times and their amidolytic activity was tested in 500 ml of the same buffer containing S-2238 (0.2 mM). Purified bovine factor X (25 $mg \cdot ml^{-1}$) was incubated at 37°C in 50 mM Tris-HCl, pH 7.8, containing 0.1 M NaCl, 10 mM $CaCl_2$ and different concentrations of the samples to be tested. Aliquots (50 ml) were removed at various times and their amidolytic activity was immediately assayed in 500 ml of the same buffer containing S-2222 (0.2 mM).

Protein C activation was assayed according to the method of Orthner et al. (1988), with minor modifications. Human protein C (5 $mg \cdot ml^{-1}$) was incubated at 37°C in 50 mM Tris-HCl, pH 7.8, containing 1 $mg \cdot ml^{-1}$ polyethylene glycol, 5 mM EDTA and dilutions of the samples to be tested. At various times, aliquots (50 ml) were taken to measure the amidolytic activity of activated protein C, in 500 ml of the same buffer containing CBS65-25 (0.3 mM).

Plasminogen activation assay

Human Glu-plasminogen (100 $mg \cdot ml^{-1}$) was incubated at 37°C in 200 ml of 50 mM Tris-HCl, pH 7.8, containing 0.1 M NaCl, 0.01% Tween-80, and different concentrations of the samples to be tested. Aliquots (50 ml) were taken at various times and assayed for plasmin activity. They were introduced into a plastic cuvette containing 450 ml of the same buffer

TABLE 2. Amidolytic activity (nmol·min^{-1}·mg^{-1}) of venoms from Chinese snakes.

Venom		Substrate					
		S-2238	S-2251	S-2222	S-2302	S-2266	CB S65-25
A. acutus	Native	230	<1	20	110	50	550
	Treated	<1	<1	<1	<1	<1	<1
A. halys	Native	30	50	70	360	290	260
	Treated	<1	<1	<1	10	40	30
T. mucrosquamatus	Native	2200	110	20	1500	2900	1600
	Treated	40	<1	<1	130	60	70
T. stejnegeri	Native	800	120	30	2400	950	1400
	Treated	10	<1	<1	240	10	10
O. hannah	Native	10	<1	10	60	50	70
	Treated	<1	<1	<1	<1	<1	<1
B. fasciatus	Native	<1	<1	<1	<1	<1	<1
B. multicinctus	Native	<1	<1	<1	<1	<1	<1
N. naja atra	Native	<1	<1	<1	<1	<1	<1

The amidolytic activities of each venom were determined as described in Methods, with the indicated substrates. Venoms were treated by PMSF as indicated in Methods, then dialyzed to remove the excess of reagent. Indicated values are the means of three determinations (standard errors were less than 10%).

supplemented with S-2251 (0.3 mM) and the formation of *p*-nitroanilide was monitored at 405 nm.

Results

Procoagulant and anticoagulant properties of the venoms

The procoagulant and anticoagulant actions of the venoms from the eight most common venomous snakes in China (Ophiophagus hannah, Naja naja atra, Bungarus fasciatus, Bungarus multicinctus, Trimeresurus stejnegeri, Trimeresurus mucrosquamatus, Agkistrodon halys and Agkistrodon acutus) were examined with human plasma in the presence and in the absence of calcium ions. Each venom was tested in its native form, after treatment with a specific and irreversible inhibitor of serine proteases (PMSF), or after treatment with a specific and irreversible inhibitor of PLA_2s (BrPBr) (Volwerk et al., 1974).

As indicated in Figure 1, *Bungarus multicinctus* venom did not modify blood coagulation *in vitro*. Venoms from *Bungarus fasciatus* and from *Ophiophagus hannah* showed a procoagulant action, dependent on the presence of Ca^{2+}. They were unable to clot purified fibrinogen (result not shown), indicating the absence of thrombin-like enzymes. Their procoagulant effect might therefore result either from an ability to convert prothrombin into thrombin in a calcium-dependent manner, or more probably, from a direct or indirect activation of factor X into factor Xa. The fact that a treatment of these venoms with PMSF significantly but not completely reduced their procoagulant action (Figure 1) suggests that this effect is due, at least in part, to serine protease(s).

The venom from *Naja naja atra* was characterized by a pronounced anticoagulant action (Figure 1). It did not prevent clotting of purified human

fibrinogen in the presence of thrombin (result not shown), indicating that the anticoagulant action is not due to fibrinogenolysis. The anticoagulant effect of *Naja naja atra* venom was completely and irreversibly prevented by treatment with BrPBr (Figure 1) which inhibited PLA_2 activity of the venom (Table 1). This activity is therefore due to one or several anticoagulant PLA_2s, as reported in the case of many other snake venoms.

The effects of venoms from Chinese *Crotalidae* snakes on blood coagulation mechanisms (Figure 2) appeared much more complex than those observed with *Elapidae* venoms (Figure 1). The venoms from *Agkistrodon acutus* and from *Trimeresurus stejnegeri* were characterized by a procoagulant action, observed both in the presence and in the absence of Ca^{2+} (Figure 2). These venoms also clotted purified fibrinogen in a calcium-independent manner (result not shown), indicating that they contain potent thrombin-like enzymes, in agreement with the observations reported by Ouyang *et al.* (1971) and by Liu and Xiong (1990). Treatment of *Agkistrodon acutus* venom with PMSF completely abolished its ability to clot purified fibrinogen (result not shown) and strongly reduced its procoagulant action (Figure 2), as expected since this effect is mainly due to thrombin-like serine proteases. However, PMSF-treated *Agkistrodon acutus* venom showed a complex action, an anticoagulant activity at 10 $mg \cdot ml^{-1}$, and a clotting activity at higher concentrations (Figure 2), suggesting that it contains other procoagulant components, in addition to thrombin-like enzymes. The anticoagulant effect of PMSF-treated *Agkistrodon acutus* venom is consistent with the presence of an anticoagulant protein of 26 kD, which is devoid of enzymatic activity and which prevents the formation of thrombin by binding to the prothrombin activation complex (Teng and Seegers, 1981). In addition to this non-enzymatic component, *Agkistrodon acutus* venom might contain anticoagulant PLA_2s, since treatment with BrPBr (Table 1) significantly increased its procoagulant effect (Figure 2).

Agkistrodon halys and *Trimeresurus mucrosquamatus* venoms were characterized by an anticoagulant action which was completely abolished after treatment with BrPBr, but not with PMSF (Figure 2). This indicates that they contain potent anticoagulant PLA_2s, as previously reported for *Trimeresurus mucrosquamatus* (Ouyang et al., 1978) and for *Agkistrodon halys* (Chen et al., 1987). In fact, treatment of *Agkistrodon halys* and *Trimeresurus mucrosquamatus* venoms with BrPBr suppressed the anticoagulant activity of these venoms and revealed a weak procoagulant activity (Figure 2), indicating the presence of procoagulant components. Further, this procoagulant action was observed only in the presence of Ca^{2+}, suggesting that the procoagulant components are able to convert prothrombin into thrombin or to activate factor X. High concentrations (100 $mg \cdot ml^{-1}$) of *Agkistrodon halys* venom were able to clot purified fibrinogen (result not shown). This may be explained by the presence of a thrombin-like enzyme, which has been purified (Guan *et al.*, 1988) but the level of this enzyme in the venom is low and its activity is weak.

Action of the venoms on purified blood coagulation factors

We determined the amidolytic activity of the venoms on a number of chromogenic substrates, classically used for assaying blood coagulation factors: thrombin (S-2238), factor Xa (S-2222), activated protein C (CBS65-25), kallikrein (S-2302 and S-2266) and plasmin (S-2251). Assays were performed with native, PMSF-treated and BrPBr-treated venoms (Table 2). The venoms from *Elapidae* snakes did not present detectable activities, with the exception of the venom from *Ophiophagus hannah* which hydrolysed several substrates (Table 2). The venoms from *Crotalidae* snakes exhibited significant activities towards most chromogenic substrates, but with important species differences: the venoms from *Trimeresurus* snakes presented much higher activities than those of *Agkistrodon* snakes and the venom from *Agkistrodon*

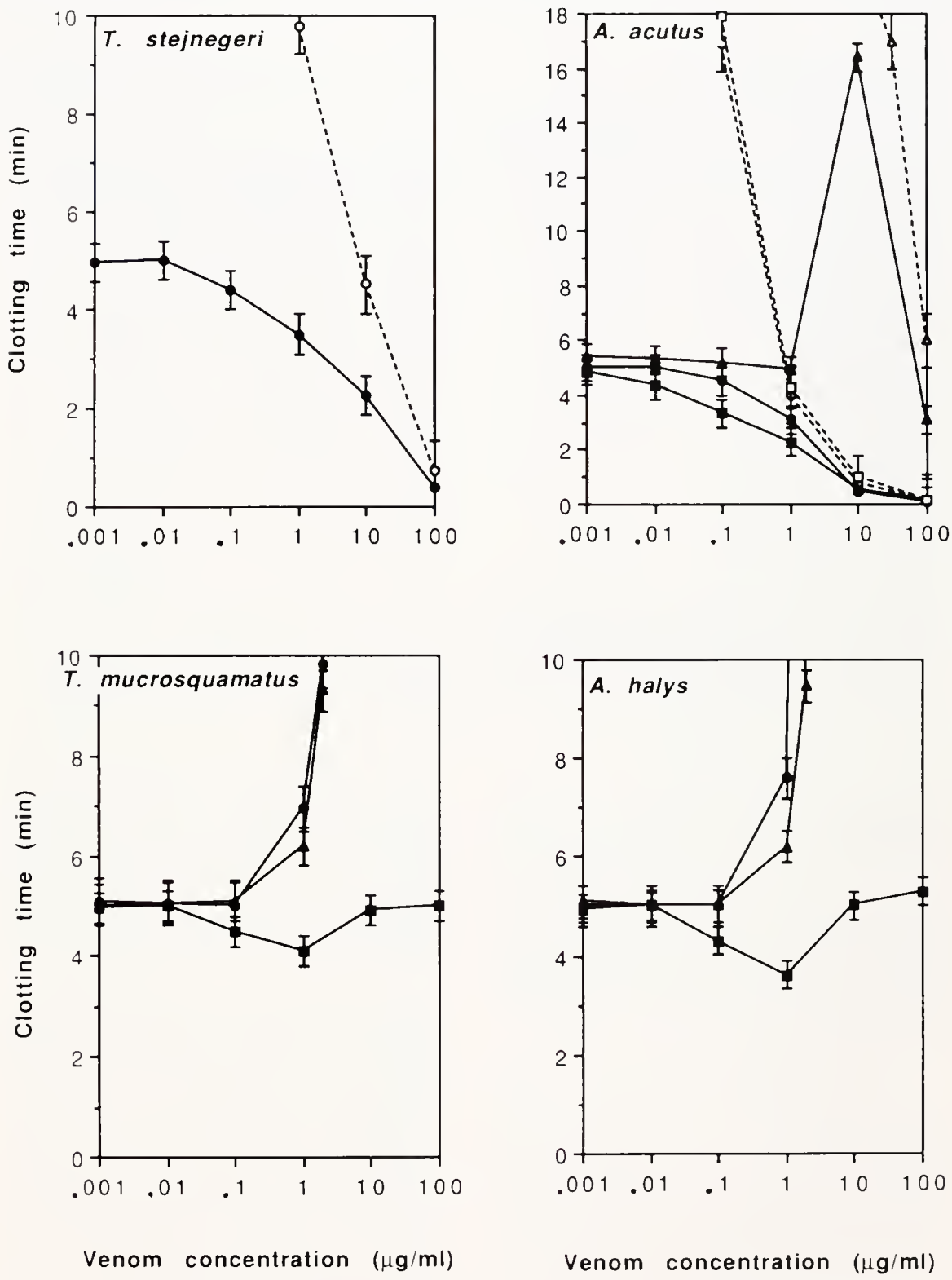

Figure 2. Effects of *Crotalidae* snake venoms on blood coagulation. Citrated platelet-poor human plasma (200 ml) was incubated at 37°C for 1 min then the sample to be tested (20 ml) was added simultaneously with calcium (5 ml of 0.45 M $CaCl_2$; 10 mM final concentration; closed symbols) or without calcium (open symbols and dashed lines). Native venom (●; ○); PMSF-treated venom (▲; △) or *p*-bromophenacyl bromide-treated venom (■; □). The values are the mean of triplicates, standard errors being 10% of the values.

TABLE 3: Activation of factor X, prothrombin, protein C and plasminogen by the venoms from common Chinese snakes.

	Activation activity (Arb. Unit)			
	Factor X	Prothrombin	Protein C	Plasminogen
A. acutus	0.67	1.3	0	0
PMSF treated	0.59	1.0	ND	ND
A. halys	0	3.0	0	0
PMSF treated	ND	2.9	ND	ND
T. mucrosquamatus	0.66	0	0	0
PMSF treated	0.58	ND	ND	ND
T. stejnegeri	0.27	0.16	0	0.42
PMSF treated	ND	ND	ND	0
Ophiophagus hannah	9.7	0	0.44	0
PMSF treated	4.2	ND	ND	ND
B. fasciatus	13.3	0	0	0
PMSF treated	5.8	ND	ND	ND
B. multicinctus	0	0	0	0
PMSF treated	ND	ND	ND	ND
N. naja	0.22	0	0	0
PMSF treated	ND	ND	ND	ND

The activation of the indicated blood coagulation factors was determined as described in Materials and Methods by measuring the amidolytic activity of the factors after activation. Each value is the mean of at least three independent determination, standard errors being less than ±10%. ND means not determined. The results were expressed as the Δ O.D./min at 405 nm acquired in analysed solution divide incubation time and divide venom concentration in activation solution.

acutus was 10 times more aetive on substrate S-2238 than that from *Agkistrodon halys*; in contrast substrate S-2251 was hydrolysed by the venom of *Agkistrodon halys* but not by the venom of *Agkistrodon acutus*. These results are in agreement with the general conceept that the proteolytic activities of the venoms from *Elapidae* snakes are much lower than those of *Crotalidae* snakes.

The proeoagulant action of the various venompurified bovine factor X, human prothrombin, protein C and plasminogen, and measuring their amidolytic activity after aetivation (Table 3). Except for *Agkistrodon halys* and *Bungarus multicinctus*, all venoms were able to activate factor X, those from *Ophiophagus hannah* and *Bungarus fasciatus* being far more active than the others. Furthermore the ability of *Bungarus fasciatus* venom to activate faetor X was markedly redueed after treatment with PMSF (Table 3), suggesting that the venom components responsible for this activity may be serine proteases. It should however be noticed that neither *Ophiophagus hannah* nor *Bungarus fasciatus* venoms showed any activity on prothrombin or plasminogen, emphasizing their rather speeific action on faetor X.

On the other hand, three venoms (*Agkistrodon acutus*, *Agkistrodon halys* and *Trimeresurus stejnegeri*) among the eight whieh have been tested, possessed componentss was further examined in detail, using which converted prothrombin into thrombin. In all eases, this activity was insensitive to PMSF, suggesting that it is not due to serine proteases. Table 3 also shows that *Ophiophagus hannah* venom was able to aetivate protein C with a low aetivity compared to that observed in the venom of *Agkistrodon contortrix contortrix* (Kisiel et al., 1987). Interestingly, the venom from *Trimeresurus stejnegeri* was eharacterized by the capaeity to activate plasminogen *in vitro* (Table 3). This action

appeared to be due to (a) serine protease(s), since it was completely abolished by PMSF.

We observed no correlation between the amidolytic activity of snake venoms, measured with the chromogenic thrombin substrate (S-2238) and their thrombin-like activity as determined by their ability to clot fibrinogen (result not shown). Similarly, there was no correlation between activation of prothrombin (Table 3) and the amidolytic activity measured with factor Xa substrate S-2222 (Table 2). These results emphasize the differences which exist between the substrate specificity of human coagulation factors and snake venom activators, and the existence in snake venoms of proteases which are able to hydrolyse chromogenic substrates without possessing the capacity to activate the corresponding blood coagulation factors.

Discussion

In the present study, we found that, except for Bungarus multicinctus, the venoms from common Chinese venomous snakes (Ophiophagus hannah, Naja naja atra, Bungarus fasciatus, Trimeresurus stejnegeri, Trimeresurus mucrosquamatus, Agkistrodon acutus and Agkistrodon halys) possessed procoagulant and/or anticoagulant activities. An in vitro analysis of these venoms indicated that their action on blood coagulation results from the combined effects of several procoagulant and anticoagulant components. In particular, comparing the effects of native venom with those of venom in which PLA_2 activity has been blocked revealed the presence of anticoagulant PLA_2s in Agkistrodon halys venom, similar to that described by Chen et al. (1987), which masked the effect of procoagulant component(s). We also showed that the procoagulant action of Agkistrodon acutus venoms does not result only from the previously described thrombin-like enzyme (Ouyang et al., 1971), but also from at least two other components, a PMSF-insensitive prothrombin activator and a calcium-dependent factor X activator. This illustrates the complexity of action of the venoms on blood coagulation mechanisms.

We demonstrated the presence of prothrombin activators in Agkistrodon acutus, Agkistrodon halys and Trimeresurus stejnegeri venoms, as well as of factor X activators in Agkistrodon acutus, Trimeresurus mucrosquamatus, Trimeresurus stejnegeri, Ophiophagus hannah, Bungarus fasciatus and Naja naja atra venoms. These studies further indicated that prothrombin and factor X activators from Crotalidae (Agkistrodon acutus, Agkistrodon halys, Trimeresurus mucrosquamatus and Trimeresurus stejnegeri) venoms are PMSF-insensitive, and Ca^{2+}-dependent in the case of factor X activators. These activators might be similar to those of Bothops atrox and Vipera russelli venoms (Kisiel et al., 1976; Hoffman and Bon, 1987a; 1987b). Interestingly, factor X activators found in Elapidae venoms (Ophiophagus hannah and Bungarus fasciatus) were inactivated after treatment with PMSF, suggesting that they might be serine proteases.

We also showed the existence of a protein C activator in the venom of Ophiophagus hannah, although its activity was low compared to that found in the venom of Agkistrodon contortrix contortrix (Kisiel et al., 1987; Orthner et al., 1988).

These studies also revealed the first evidence of the existence of a plasminogen activator. Trimeresurus stejnegeri contains a PMSF-sensitive plasminogen activator. Its biochemical structure and mechanism of action are currently under investigation.

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