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Anticoagulant and factor Xa-like activities of *Tityus discrepans* scorpion venom

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Abstract. *Tityus discrepans* venom (*TdV*) produces a variety of haemostatic manifestations including alveoli fibrin deposition and/or prothrombin and partial thromboplastin time (PT, PTT) alterations in mammals. *In vitro* studies have demonstrated that *TdV* contains tissue plasminogen activator-like (t-PA), fibrinolytic and plasmin inhibitory compounds and produces platelets activation through GPVI and a novel Src-dependent signalling pathway. The aim of this study is to describe the initial characterization of procoagulant and anticoagulant components from *TdV*. This venom was fractionated by exclusion molecular chromatography on a Sephadex G-50 column. The eluted material was collected as five fractions called S1 to S5. These fractions and the whole venom were used to evaluate factor Xa- and thrombin-like activities, fibrinogen degradation, furthermore thrombin- and factor Xa-inhibitory activities. The results demonstrated that *TdV* contain components with factor Xa-like activity (procoagulants) as well fibrinogenolytic compounds present in the fraction S1 and components with factor Xa inhibitory activity in the fractions S4 and S5 (anticoagulants).

Keywords: Scorpion; Coagulation; Fibrinogen; Factor Xa.

Resumen. El veneno de *Tityus discrepans* (*TdV*) produce en mamíferos una variedad de manifestaciones hemostáticas tales como depósitos de fibrina en alveolos y/o alteración en los tiempos de protrombina y tromboplastina parcial (PT, PTT). Estudios *in vitro* han demostrado que el *TdV* contiene componentes semejantes al activador del plasminógeno tipo tisular (t-PA), fibrinolíticos, compuestos que inhiben la actividad de plasmina y además componentes que promueven la activación de plaquetas a través del receptor GPVI y por una nueva vía de señalización dependiente de las Src kinasas. El objetivo de este estudio es describir la caracterización inicial de componentes procoagulantes y anticoagulantes a partir del *TdV*. Este veneno fue fraccionado por cromatografía de exclusión molecular sobre una columna Sephadex G-50. El material eluido fue colectado en cinco fracciones denominadas S1 a S5. Estas fracciones y el veneno completo fueron usados para evaluar actividades semejantes a factor Xa y trombina, degradación de fibrinógeno, como también la inhibición de la actividad del factor Xa y de la trombina. Los resultados demostraron que *TdV* contiene componentes con actividad semejante al factor Xa (procoagulantes) y compuestos fibrinogenolíticos presentes en la fracción S1, además de componentes con actividad inhibitoria del factor Xa presentes en la fracción S4 y S5 (anticoagulantes).

Palabras claves: Escorpión; Coagulación; Fibrinógeno; Factor Xa.

INTRODUCTION

Many animal venoms act on the human hemostatic system as procoagulants or anticoagulants. The procoagulant components cause activation of the coagulation system and can induce a massive consumption of coagulant factors (Markland 1998a; 1998b; Arocha-Piñango *et al.* 1999a; 1999b; Reis *et al.* 2001; Flores *et al.* 2006); also some components promote pro-coagulant response of platelets by GPVI receptor activation such as *Tityus discrepans* venom (*TdV*) (Brazón *et al.* 2011). However, anticoagulant components may inhibit coagulant enzymes such as factor Xa or thrombin (Markward 1994; Baskova and Zavalova 2001; Basanova *et al.* 2002; Motoyashiki *et al.* 2003). Venoms may also cause an anticoagulant effect due to fibrinogen or fibrin degradation related with serine- or metalloproteases (Markland 1998a; 1998b; Pinto *et al.* 2004; Serrano and Maroun 2005; Swenson and Markland 2005).

Tityus discrepans venom contains anticoagulant and procoagulant components which produce alterations of the partial thromboplastin time (PTT) and prothrombin time (PT) (Brazón *et al.* 2008). Furthermore, discreplasminin an antifibrinolytic compound has been isolated from *TdV* (Brazón *et al.* 2009). The purpose of this study is to describe the initial characterization of procoagulant and anticoagulant components from *TdV*. In this paper, we evidenced the presence of procoagulant components showing factor Xa-like activity, while the anticoagulant components inhibited the factor Xa activity and degraded the fibrinogen molecule.

MATERIALS AND METHODS

Animals, venom and purification

Tityus discrepans scorpions were collected in the areas surrounding Caracas, Venezuela. Adult scorpions (~100) were kept alive in a laboratory with food and water *ad libitum*. Venom was extracted from them once a month. The animals were immobilized with CO_2 and the venom extraction was by electrical stimulation with pulse 50 V at 60 Hz for 100 milliseconds. The venom was dissolved in double distilled water and centrifuged at 15,000 g for 15 min at 4 °C to remove the insoluble materials. The supernatant was lyophilized and stored at – 80 °C until used. The protein concentration was estimated by spectrophotometry assuming that 1 unit of absorbance/cm of path length at 280 nm corresponds to 1 mg protein/ml (D´Suze *et al.* 1996). This venom was fractionated by exclusion molecular chromatography on a Sephadex G-50 column (1x200 cm) equilibrated and eluted with 20 mM ammonium acetate, pH 4.7, with a flow rate of 0.25 ml/min. Proteins were detected at 280 nm.

Anticoagulant activity

The fibrinogenolytic activity described by Salazar *et al.* (2007) was used to determine the anticoagulant activity of crude venom and fractions. The human fibrinogen solution (in 0.05 M imidazole-0.15 M NaCl, pH 7.4 buffer) was incubated with *TdV* for 24 h at 37 °C, at 0.1/30; 0.25/30; 0.5/30; 1/30 and 5/30 µg venom/µg fibrinogen ratios. Fibrinogen degradation (molecule or chains) was visualized by SDS-PAGE under non-reducing or reducing conditions, respectively, using Tris-Tricine-system on 5 and 7.5% gel (with 49.5% acrylamide/3% bisacrylamide) (Schagger and Jagow 1987).

Residual coagulant activity of fibrinogen pretreated with *TdV* or fraction S1, was determinate by the thrombin time method (TT) (Austen and Rhymes 1975). Briefly, a mixture of 0.1 ml of 0.05 M Tris-HCl buffer, pH 7.4 (coagulation buffer) plus 0.1 ml of 0.3 % human fibrinogen (in coagulation buffer) treated or not with venom or fraction S1 for 24 h at 37°C and at a 1/30 ratio, was incubated in a borosilicate tube (10x75 mm). Then, 0.1 ml of thrombin solution (in coagulation buffer-2.5 IU/ml) was added, thoroughly mixed at 37°C and clotting time recorded in seconds. Tests were performed three times and the median clotting time calculated. The results were expressed in seconds.

Anticoagulant effect of *Tityus discrepans* also was evaluated using factor Xa or thrombin as substrates. The enzymes, factor Xa (0.3 nKcat/µl) or thrombin (0.01 IU/ml) were incubated at 37 oC for 30 min with different concentrations of *TdV*, S1 to S5 fractions. Then, the amidolytic activity at 405 nm was evaluated following the method of Guerrero *et al.* (1992), using S-2222 or S-2238 chromogenic substrate (0.8 mM and 0.6 mM, final concentration), respectively. The activity was expressed as UA/min/µg. The enzymes incubated with buffer were used as positive controls.

Procoagulant activity

Tityus discrepans venom coagulant activity was evaluated using S-2222 and S-2238 chromogenic substrates, which determined the factor Xa or thrombin-like activities. A mixture of 80 µl of 50 mM Tris-HCl buffer, pH 8.4, 10 µl of sample venom (1 - 10 µg/µl) and 10 µl of substrate (0.8 mM S-2222 or 0.6 mM S-2238, final concentration) were placed in each well of 96 well polystyrene plates. After incubating at 37 °C for 30 min, the absorbance at 405 nm was measured. One unit of amidolytic activity was expressed as UA/min/µg. Furthermore, factor Xa-like amidolytic activity was assayed in the presence of protease inhibitors. Prior to the assay, *TdV* or its fractions were incubated for 30 min at 37 °C, with each protease inhibitors using 10 mM 1,10 phenanthroline, 10 mM ethylenediaminetetraacetic acid (EDTA-Na), or 10 mM bis(2-aminoethyl) tetraacetic acid (EGTA-Na) as metalloprotease inhibitors; 10 mM benzamidine, 1 µg/µl soybean trypsin inhibitor (SBTI) or 100 UI/ml aprotinin as serine protease inhibitors; and 0.1 mM iodoacetic acid as cysteine protease inhibitors.

Statistical analysis

Data were processed using nonparametric statistical procedures. Data were expressed as medians and their 90% confidence intervals (CI) with n=3, calculated by Hollander and Wolfe 1973.

RESULTS

Tityus discrepans venom was fractionated on Sephadex G-50 column and the eluted material was collected as five fractions called S1 to S5 (*Figure 1A*).

The proteases present in *TdV* induced a slight increase of fibrinogen electrophoretic mobility when a *TdV*/fibrinogen ratio ≥ 0.5/30 was used; this effect was greater at a ratios ≥ 1/30. At the same conditions was also evidenced a fibrinogen Aα-chains degradation; whereas Bβ and γ chains were unaffected (not shown). The ratio 1/30 was used to evaluate the effect of *TdV* on fibrinogen molecule/chains at different incubation times. An increase in electrophoretic mobility of fibrinogen at times ≥ 16 h was observed. Fibrinogen Aα chains degradation started after 2 h of incubation with *TdV* and the effect was completed 24 h later. The Bβ and γ chains degradation did not was observed in these conditions (not shown). Fibrinogen incubation for 24 h at 37 °C with *TdV* or with Sephadex G-50 fraction S1 (ratio 1/30), under non reducing conditions produced an increase in fibrinogen electrophoretic mobility (*Figure 1B*, lanes 2 and 3). Under reducing conditions, *TdV* only degraded fibrinogen Aα-chains, however fraction S1 degraded both Aα and Bβ chains, generating degradation fragments with molecular weights < 31 kDa (*Figure 1C*, *lanes 2 and 3*). S2 to S5 fractions had no effect on fibrinogen molecule or chains (*Figure 1B and 1C*).

The fibrinogen coagulant activity residual after treating with either *TdV* or fraction S1 (at a 1/30 ratio) was evaluated using the TT. The results showed that TT is prolonged as the fibrinogen incubation time with *TdV* or fraction S1 increases (not shown).

In relation to factor Xa or thrombin amidolytic activity, the results showed that hundred micrograms *TdV* increased the factor Xa activity (0.3 nKcat/µl) from 758.70 to 864.93 (864.92-864.96) UA/min/µg and this effect was more pronounced with fraction S1 (100 µg), observing an amidolytic activity increase from 758.70 to 998.73 (998.71- 998.77). In contrast, fractions S4 and S5 inhibited the factor Xa activity in 56.11 and 16.17%, respectively (*Table 1*). *TdV* did not modify the thrombin amidolytic

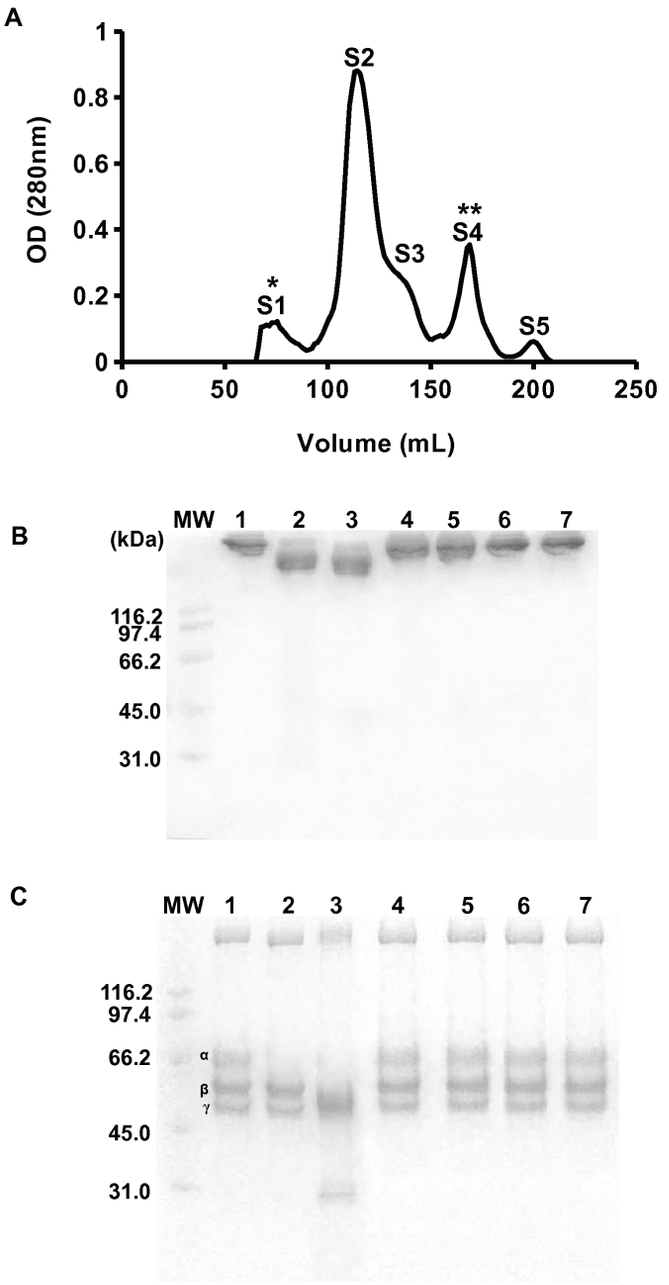

Figure 1. Tityus discrepans venom (TdV) chromatography and fibrinogenolytic activity of TdV and its Sephadex G-50 fractions. (A) *TdV* (100 mg/ml) was passed through a Sephadex G-50 column (1 x 200 cm), with 20 mM of ammonium acetate, pH 4.7, detecting at OD 280 nm with a 0.25 ml/min flow rate. Five fractions were obtained (S1-S5). (B) Fibrinogen molecule degradation (30 µg) by *TdV* (1 µg) or by its fractions (1 µg) after 24 h incubation at 37 °C was visualized by SDS-PAGE using Tris-Tricine-system on 5% gel under non reducing conditions. (C) Fibrinogen chains degradation (30 µg) by *TdV* (1 µg) or by its fractions (1 µg) after 24 h incubation at 37 °C was visualized by SDS-PAGE using Tris-Tricine-system on 7.5% gel under reducing conditions. 1.- Fibrinogen (Fg) control. 2.- Fg + *TdV*. 3.- Fg + S1. 4.- Fg + S2. Fg + S3. Fg + S4. Fg + S5. Fibrinogen consists of three polypeptide chains Aα, Bβ and γ. Standard proteins were used as a reference to determine the molecular weight. The gels were stained with R-250 Coomassie Blue.

activity (data not shown). These results suggested the existence in *TdV* of a factor Xa-like activity and a factor Xa inhibitory activity which are present in the fraction S1 (procoagulant activity) and S4 and S5 (anticoagulant activity). The results also evidenced a direct amidolytic activity with *TdV* due to 10 µg crude venom hydrolyzed S-2222 substrate with an amidolytic activity of 19.80 (19.79-19.81) UA/min/µg (*Table 1*). This effect was more pronounced with 100 µg crude venom and the activity was of 106.23 (106.23 - 106.24) UA/min/µg. Furthermore, the amidolytic activity was enriched four times in fraction S1 (*Table 1*). Fractions S2 - S5 had no hydrolyzed the S-2222 substrate (Table 1). This amidolytic activity was reduced by > 58% in presence of serine protease inhibitors such as benzamidine or SBTI (factor Xa specific inhibitor) (*Table 1*). In contrast, EDTA, EGTA, phenanthroline, iodoacetic acid or aprotinin did not modify this activity (data not shown).

Table 1. Factor Xa-like and Factor Xa inhibitory activities of *TdV* and its Sephadex G-50 fractions. Factor Xa-like activity was measured by an amidolytic micromethod (S-2222 substrate) in presence or absence of protease inhibitors. To evaluate the effect of *TdV* or its fraction on factor Xa activity was incubated for 30 min at 37°C factor Xa (0.3 nkat/µl) with *TdV* or Sephadex-G50 fractions. Data were expressed as median and its IC at 90% confidence, n = 3, ND = No determined. Benz: Benzamidine.

Action of *TdV* and its fractions on factor Xa activity

Sample (10 µL)	Amidolytic activity (S-2222) UA/min/µg	% inhibition
FXa (0.3 nKcat/µL)	758.70 (758.69-758.72)	0.00
FXa + TdV (10 µg)	545.64 (545.63-545.66)	28.08
FXa + TdV (50 µg)	670.63 (670.62-670.65)	11.61
FXa + TdV (100 µg)	864.93 (864.92-864.96)	0.00
FXa + S1 (100 µg)	998.73 (998.71-998.77)	0.00
FXa + S4 (100 µg)	333.00 (332.98-333.10)	56.11
FXa + S5 (100 µg)	636.02 (636.00-636.04)	16.17

Factor Xa-like activity

Sample (10 µL)	Amidolytic activity (S-2222) UA/min/µg	% inhibition	
		Benz	SBTI
TdV (10 µg)	19.80 (19.79-19-81)	N.D	N.D
TdV (50 µg)	78.60 (78.59-78.63)	N.D	N.D
TdV (100 µg)	106.23 (106.23-106.24)	65.63	58.60
S1 (100 µg)	404.08 (404.08-404.10)	88.50	78.41

In addition, nor *TdV* neither the chromatographic fractions showed thrombin-like amidolytic activity.

DISCUSSION

Scorpions using their venom to defend themselves from predators or to capture their prey; their venoms are complex mixtures of biologically active components over different types of organisms, such as bacteria, insects, fungi and mammals (D'Suze *et al.* 2004a; 2004b; Díaz *et al.* 2009; Joya *et al.* 2011). Scorpion venoms

are generally not known to have the ability to produce hemostatic alterations. However, there are exceptions as *Centruroides sculpturatus* (Watt et al., 1974; Longenecker and Longenecker 1981), *Buthus tamulus* (Devi *et al.* 1970; Reddy *et al.* 1972), *Buthus martensii* (Song *et al.* 2005) and *Tityus discrepans* (D´Suze *et al.* 2003; 2004b; Brazón *et al.* 2008; 2009; 2011) that their venoms, in case of severe envenoming perturb the mammal hemostatic system. The relevance of components with action on haemostatic system (platelet, coagulation and fibrinolysis) in scorpion venoms is not clear. However, the presence of these components has pathophysiologic implications severe in the scorpion envenoming.

The results of fibrinogenolytic activity indicate that *TdV* contains fibrinogenases that induce fibrinogen degradation associated with an anticoagulant effect. Similar components were isolated from snake venoms, such as ancrod, this compound is medically important as therapeutic agents (Swenson and Markland 2005). On the other hand, the results of this study indicated the presence of anticoagulant components with factor Xa inhibitory activity in *TdV*, which are responsible of the previously described PTT and PT prolongation (Brazón *et al.* 2008).

Furthermore, this venom produces a factor Xa-like activity (procoagulant activity) which was found in fraction S1; this finding is consistent with the synergism in amidolytic activity observed when commercial factor Xa was incubated with either *TdV* or fraction S1. Probably, the components with the factor Xa-like activity present in the venom and S1 induced the previously described PTT shortening (Brazón *et al.* 2008). The benzamidine or SBTI effects on the venom amidolytic activity, supports the notion that a serineproteases with factor Xa-like activity exists in T. discrepans venom. Additionally, the *TdV* did not hydrolyze the S-2238 substrate. These results indicate the absence of thrombin-like activity in venom (data not shown).

We have shown that *TdV*, in addition to t-PA-like compounds and discreplasminin, a plasmin inhibitor (Brazón *et al.* 2009), also contains anticoagulant compounds able to inhibit factor Xa amidolytic activity and to induce fibrinogen degradation. Our results also demonstrated the existence of a procoagulant activity which was similar to factor Xa. All these compounds, per se or in combination, could explain the alterations of PT and PTT (Brazón *et al.* 2008) as well as the fibrin deposits observed in lung alveoli from experimentally envenomed mammals (D'Suze *et al.* 2004b).

The most dangerous complication induced by *TdV* is the "scorpion venom respiratory distress syndrome (SVRDS)". Our group has demonstrated that this complication started by neutrophil lung infiltration and sequestration, with important fibrin deposition as the last step of a cascade reaction where leukocytes activation products are involved (D'Suze *et al.* 2003). However the pathophysiology of SVRDS is complex and cannot be entirely attributed to the unregulated leukocytes activation products, SVRDS may in part be associated with the procoagulant components found in *TdV*. These are not abundant, < 1% of the venom, but are able to produce coagulation disorders both in scorpionism patients as well as *in vitro* studies (Brazón *et al.* 2008; D'Suze *et al.* 2003, 2004). Perhaps venom procoagulant components act in a synergistic form with neutrophil procoagulant products enhancing fibrin clots formation. These critical questions wait for answers from ongoing studies.

This investigation has motivated the further purification and physiological characterization of the compounds responsible of the haemostatic alterations reported here. These compounds are very interesting due its biotechnological applications on clinical disorders associated with the fibrinolytic system and coagulation, it is mandatory to determine their action mechanisms, physiological effects and pharmacokinetic parameters.

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