

# Fibrinogenolytic activity of venom proteins of *Montivipera xanthina* (Gray, 1849) (Ophidia: Viperidae)

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In this study, with the aim of evaluating coagulant activities in the venom of *Montivipera xanthina*, we analyzed venom proteins, digestion patterns of fibrinogen chains incubated with venom, and the effects of protease inhibitors on *M. xanthina* venom proteases. Venom samples were obtained from four adult specimens collected in Gümlüdü (Izmir, Turkey). SDS-PAGE analysis showed the presence of 17 protein bands or band groups in the molecular mass range of 20 to 200 kDa. The specific digestion patterns of fibrinogen chains revealed that *M. xanthina* venom possesses fibrinogenolytic enzymes, which could be involved in coagulation processes during envenomation. Fibrinogenolytic activity affected the A $\alpha$ -chain and showed a time-dependent effect on B $\beta$ -chains, which suggests the presence of both metalloproteinases and serine proteases in *M. xanthina* venom. After observing the fibrinogenolytic activity of *M. xanthina* venom, further research should focus on the isolation, identification, and characterization of individual venom components in order to provide insight into their function and biological roles.

**Key words:** *Montivipera xanthina*; tris-tricine SDS/PAGE; venom; Viperidae.

Snake venoms contain numerous toxic and non-toxic proteins and peptides with many biological activities (CHIPPAUX & GOYFFON, 1998). Viperid venoms often lead to inflammation, prominent local oedema and necrosis. In addition to these local symptoms, a more complicated and species-specific envenomation symptoms including systemic and local bleeding, intravascular coagulation and shock, is triggered by individual and synergetic interactions of active venom components (GUTIÉRREZ & LOMONTE, 1989, 1995; TENG & HUANG, 1991; WARRELL, 2005).

The majority of viperid venom components that are responsible for enven-

omation symptoms are proteins and peptides that have highly specific functions. While hydrolases, L-amino acid oxidases, phospholipases, thrombin-like procoagulants, kallikrein-like serine proteases and metalloproteinases (13-150 kDa) constitute 80-90% of viperid venom solutes (MACKESSY, 2010), small polypeptide toxins (5-10 kDa) have been also noted (LAURE, 1975; FOX *et al.*, 1979; BIEBER & NEDELHOV, 1997; CARBAJO *et al.*, 2015). To date, a wide number of proteins and polypeptides belonging to 20 venom protein families have been identified and characterized from the venoms of several viperid snake species (GITTER *et al.*, 1957, 1959; PERKINS *et al.*,

1993; PERKINS & TOMER, 1995; SANZ *et al.*, 2006, MACKESSY, 2010). The predominant presence of enzymatic components, especially hydrolytic enzymes, in viperid venoms has been documented by many proteomic studies (NAWARAK *et al.*, 2003; SERRANO *et al.*, 2005; SANZ *et al.*, 2006, 2008; ANGULO *et al.*, 2008; MACKESSY, 2010).

Although the general pattern of venom composition in Viperidae is well known, intra- and interspecific variations in venom components depending on age, sex, season, diet, geographical origin and many other factors, have been reported (THEAKSTON & REID, 1978; MEIER & FREYVOGEL, 1980; CHIPPAUX *et al.*, 1991; TUN-PE *et al.*, 1995; ARIKAN *et al.*, 2006, 2014; VONK *et al.*, 2011). Even though there are numerous studies on venom components and the variations they exhibit, studies of mechanisms underlying this variation are limited (EARL *et al.*, 2006).

A large number of fibrinogenolytic enzymes that induce alterations in the blood coagulation cascade have been isolated from the venoms of Viperidae snake species and characterized (MARKLAND, 1998; SWENSON & MARKLAND, 2005). These metalloproteinases that are not inhibited by human serum proteinase inhibitors cleave preferentially the A $\alpha$ - or B $\beta$ -chain of fibrinogen (SWENSON & MARKLAND, 2005).

The Ottoman viper, *Montivipera xanthina* (Gray 1849), is distributed from north-eastern Greece, through some of the Aegean islands, to western, southern and central Anatolian Turkey up to 2000 m (BARAN & ATATÜR, 1998; SINDACO *et al.*, 2013). Although pathological effects of *M. xanthina* venom on different tissues have been reported for both animals and humans

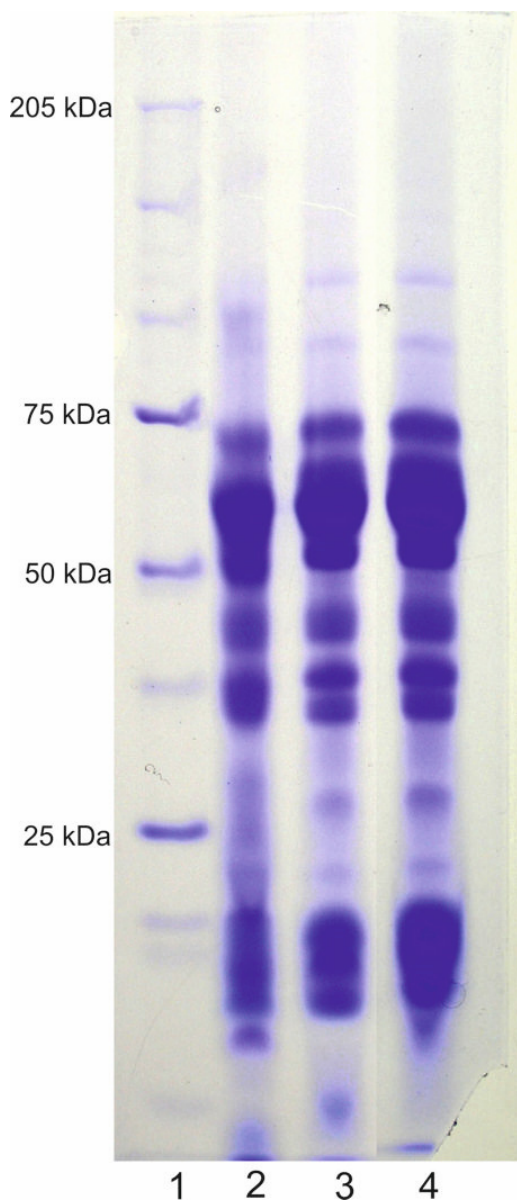
(BOZKURT *et al.*, 2008; CESARETLI & ÖZKAN, 2010; TOPYILDIZ & HAYRETDAG, 2012), detailed descriptions of venom components and their biological activities are relatively limited (BERNADSKY *et al.*, 1986; TAN & PONNUDURAI, 1990; ARIKAN *et al.*, 2003, 2005, 2006; NALBANTSOY *et al.*, 2013; YALÇIN *et al.*, 2014). Furthermore, fibrinogenolytic activity of *M. xanthina* venom has not yet been studied.

In this study, with the aim of evaluating coagulant activities in the venom of *M. xanthina*, protein bands, and the hydrolysis of fibrinogen chains caused by venom, were analysed using Tris-Tricine SDS-polyacrylamide gel electrophoresis. In addition, effects of protease inhibitors on *M. xanthina* venom proteases were also studied.

## MATERIALS AND METHODS

*Montivipera xanthina* samples used in this study (two males and two females) were collected in Gümlükdür (Izmir, Turkey). All venoms were obtained from individual snakes according to TARE *et al.* (1986), without applying pressure on venom glands. Venom samples were lyophilized and kept at -20°C prior to electrophoretic analysis.

Venom samples were dissolved in 0.1 M Tris-HCl buffer containing 0.5 mM CaCl<sub>2</sub> and 0.01% NaN<sub>3</sub> (pH 8.0, 250 µg / ml) and were centrifuged at 500 xg during 10 minutes. The resulting supernatant, which has a light yellow colour, was used as venom sample in further analysis. Protein concentration was determined in venom samples in triplicate with Coomassie Blue according to the Bradford method (BRADFORD, 1976), with a sensitivity between 5 and 100 µg protein ml<sup>-1</sup>. For this process, 5 µl of venom sample were diluted

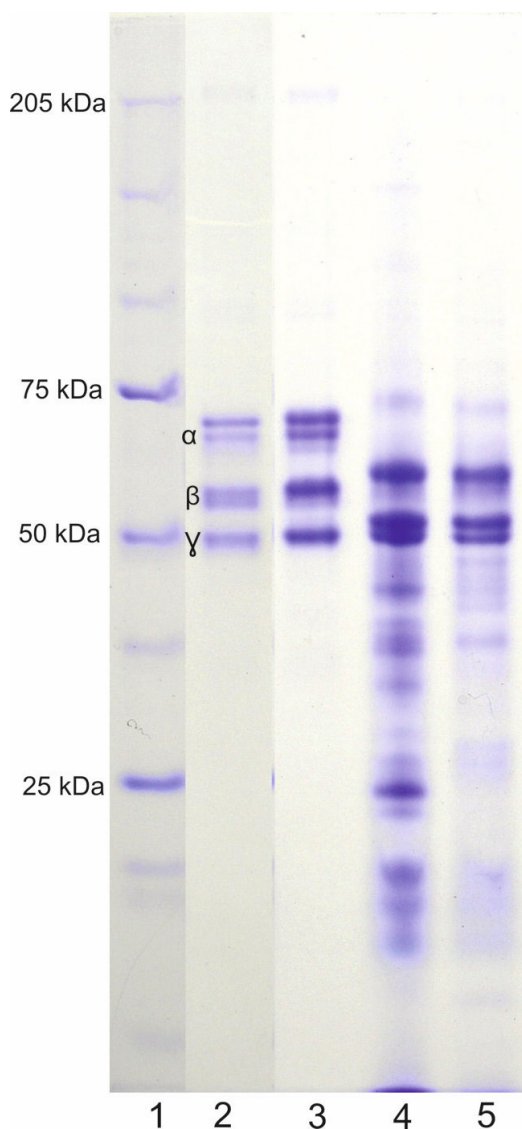


**Figure 1:** Protein bands of *Montivipera xanthina* venom on a 10% Tris-Tricine SDS/PAGE under non-reducing and reducing conditions. Well 1 corresponds to molecular mass markers, well 2 to denatured venom in non-reducing conditions (without 2-mercaptoethanol), and wells 3 and 4 to denatured venom in reducing conditions (with 2-mercaptoethanol).

ed with 95  $\mu$ L ultra-pure water (dilution factor 1:20) and incubated at room temperature for 5 to 45 minutes with Bradford Reagent (Sigma B6916). Bovine serum albumin (BSA-Sigma) was used as a standard, and absorbance values of the samples were determined at 595 nm.

Separation of venom proteins was carried out with Tricine-sodium dodecyl sulphate (TSDS) polyacrylamide gel electrophoresis (PAGE) (SHÄGGER & VON JAGOW, 1987), which ensures the separation of polypeptides with a molecular mass  $\leq 2$  kDa (SHI & JACKOWSKI, 1988). Electrophoretic separations were carried out on a discontinuous buffer system using a 10% separation gel and a 4% stacking gel (cathode buffer: 0.1 M Tris, 0.1 M Tricine, 1% SDS, pH 8.25; anode buffer: 0.2 M Tris-HCl, pH 8.9). Five  $\mu$ L of venom sample were loaded onto gels after being denatured in a sample buffer that contained 100% glycerol, 2-mercaptoethanol, 20% SDS, and 1M Tris, pH 6.8 for 5 minutes at 95°C. Electrophoresis were maintained for 14 hours with 25 mA stable current using a SE Ruby 600 (Amersham Bioscience, Piscataway, New Jersey, USA) apparatus with gels having 18 x 16 x 0.15 cm dimension. Gels were then stained with 0.1 % Coomassie Blue R-250 (Sigma) for 3 h. Wide range standards (6.5-205 kDa) (Sigma) were used for molecular mass determinations.

Hydrolysis of fibrinogen by *M. xanthina* venom was shown by Tris-Tricine SDS gel electrophoresis (SHÄGGER & VON JAGOW, 1987) on 10% polyacrylamide gels. Human fibrinogen (type I, Sigma) was dissolved in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 mM  $\text{CaCl}_2$  and 0.02%  $\text{NaN}_3$  at a final concentration of 5 mg / ml. Briefly, 100  $\mu$ L



**Figure 2:** Tris-tricine SDS-PAGE analysis of human fibrinogen after digestion by *Montivipera xanthina* venom in a 10% gel under reducing conditions. Well 1 corresponds to molecular mass markers, well 2 to human fibrinogen control incubated at 37°C for 10 minutes without venom, and wells 3, 4 and 5 to human fibrinogen samples after incubation at 37°C with venom at a ratio 20:1 (w/w) for 10, 60 and 120 minutes, respectively.

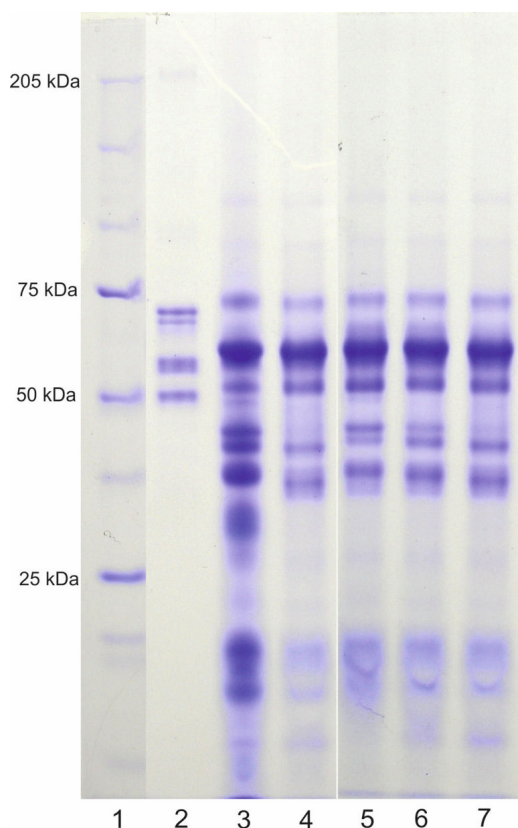
fibrinogen solution was incubated with an equal volume of venom sample (250 µg / ml) at 37°C, corresponding to a ratio of 20:1 (w/w). Time-dependent hydrolysis of fibrinogen with venom was performed at 10, 60 and 120 minute incubation times. The samples were denatured and reduced at 95°C for 5 minutes in 1.0 M Tris-HCl buffer (pH 6.8) containing glycerol, 1% 2-mercaptoethanol, and 4% SDS before electrophoresis.

Effects of protease inhibitors on *M. xanthina* venom proteases were investigated using Na<sub>2</sub>EDTA and a protease inhibitor cocktail containing a broad spectrum of serine, cysteine and metalloprotease inhibitors (Protease Inhibitor Cocktail Tablets, Roche). For inhibition studies, venom samples were incubated with 45 mM Na<sub>2</sub>EDTA or protease inhibitor cocktail in duplicate at room temperature for 1 hour before incubation with fibrinogen.

## RESULTS

The average total protein content of *M. xanthina* venom extracts was estimated as 145 mg / ml. A total of 17 protein bands or band groups in the range of 20 to 200 kDa were detected with 10% Tris-Tricine PAGE after denaturation under non-reducing and reducing conditions (Fig. 1). Most of the proteins from venom secretions displayed intensively in the range between 25 and kDa. Moreover, a dense fraction group lighter than 25 kDa and some fractions having lower molecular masses were also observed.

The human fibrinogen Aα-chain (63 kDa) was completely hydrolyzed after 60 minutes of incubation with *M. xanthina* venom (Fig. 2). After 60 and 120 minutes



**Figure 3:** Effect of the protease inhibitors on the digestion of fibrinogen by *Montivipera xanthina* venom in a 10% gel under reducing conditions. Well 1 corresponds to molecular mass markers, well 2 to human fibrinogen control incubated at 37°C for 10 minutes without venom, wells 3 and 4 to human fibrinogen incubated at 37°C with venom and Na<sub>2</sub>EDTA for 10 and 120 minutes, respectively, and well 5, 6 and 7 to human fibrinogen incubated at 37°C with venom and protease inhibitor cocktail for 10, 60 and 120 min, respectively.

of incubation, a weak band of B $\beta$ -chain (56 kDa) and prominent protein bands of 60 and 52 kDa were observed. Beginning at minute 60 of incubation, several fractions lighter than 52 kDa also appeared. While most of these light fractions were almost

completely hydrolyzed after 120 min, the fibrinogen  $\gamma$ -chain and fractions of 60 and 52 kDa were stable (Fig. 2).

Metalloprotease inhibitor Na<sub>2</sub>EDTA alone did not inhibit fibrinogenolytic activity (Fig. 3). After 10 minutes of hydrolysis in the presence of Na<sub>2</sub>EDTA, only a weak band of A $\alpha$ -chain and several fractions of 46, 38, 31, 27 and 22 kDa were observed. After 120 minutes of hydrolysis in the presence of Na<sub>2</sub>EDTA, while fractions of 46, 38 and 22 kDa had almost completely disappeared, B $\beta$ - and  $\gamma$ -chains were stable. On the contrary, fibrinogenolytic activity was partially inhibited with the protease inhibitor cocktail containing serine, cysteine, and metalloproteinase inhibitors (Fig. 3). After 10 and 60 minutes of hydrolysis in the presence of the protease inhibitor cocktail, a weak band of A $\alpha$ -chain and three fractions of 38, 31 and 27 kDa were observed. After 120 minutes of hydrolysis in the presence of the protease inhibitor cocktail, the fraction of 38 kDa had almost completely disappeared.

## DISCUSSION

The electrophoretic profile of *M. xanthina* venom is generally similar to electrophoretic profiles of viperid venoms (GITTER *et al.*, 1957, 1959; AROCH & HARRUS, 1999; BERNADSKY *et al.*, 1986; TAN & PONNUDURAI, 1990; MACKESSY, 2010). Typical protein families found in viperid venoms are nucleases and L-amino acid oxidases (150-160 kDa), metalloproteinases P-III (55-60 kDa), serine proteases (40-50 kDa), CRISPs (21-25 kDa), metalloproteinases P-I (16-20 kDa), PLA<sub>2</sub>s and snaclecs (10-15 kDa), disintegrins (6-10 kDa) and myotoxins (~6 kDa) (MACKESSY, 2010).

Viperid venoms are characterized by prominent presence of high molecular mass components, primarily hydrolytic enzymes, and serine proteases dominate the mid-mass ranges (~28-36 kDa). Snake venom metalloproteinases and disintegrins are responsible for major local symptoms in snakebites, including haemorrhage, oedema, hypotension, inflammation, and necrosis (HUANG, 1998; GUTIÉRREZ *et al.*, 2009; VONK *et al.*, 2011). The metalloproteinases are most often a dominant component of viperid venoms (SANZ *et al.*, 2006; CALVETE *et al.*, 2007), being the major protein family involved in digestion of the prey (THOMAS & POUGH, 1979; MACKESSY, 1998). P-I metalloproteinases, which do not cause haemorrhage, are fibrinolytic agents (MARKLAND, 1998; Hsu & HUANG, 2010). Based on their specificities, fibrinogenolytic proteases are classified as  $\alpha$ - or  $\beta$ -chain fibrinogenases (SWENSON & MARKLAND, 2005). Thrombin-like serine proteinases of snake venom (TL-SVSP) deplete fibrinogen stores by producing micro-clots (STOCKER *et al.*, 1982; MARKLAND, 1998; MACKESSY, 2010; SÁNCHEZ *et al.*, 2010) that are readily destroyed by the prey's anti-clotting machinery.

SDS-PAGE analysis of fibrinogen in the presence of venom revealed that *M. xanthina* venom possesses fibrinogenolytic enzymes that specifically cleave the A $\alpha$ -chain and B $\beta$ -chains of fibrinogen (Fig. 2). Fibrinogenolytic activity affected mostly the A $\alpha$ -chain, had a time-dependent effect on B $\beta$ -chains and did not affect at all the  $\gamma$ -chain, all of which suggests the presence of both metalloproteinases and serine proteases. Similar fibrinogenolytic activity patterns have been also reported for ven-

oms of several other viperid species (Tu *et al.*, 1996; RETZIOS & MARKLAND, 1994; SIIGUR *et al.*, 1998; RODRIGUES *et al.*, 2000; RAMOS *et al.*, 2003; LEONARDI *et al.*, 2007). The absence of specific activity of the venom on the  $\gamma$ -chain of fibrinogen also suggests that *M. xanthina* venom fibrinogenases do not activate plasminogen leading to plasmin formation, which cleaves peptide bonds at the carboxy-terminal side of lysine residues in the A $\alpha$ -, B $\beta$ - and  $\gamma$ -chains of fibrinogen (MARKLAND, 1998).

Although several new small bands were observed supposedly because of fibrinogen hydrolysis over time (Fig. 2), it is difficult to differentiate the degradation products of fibrinogen subunits from venom components. For definitive identification, it is necessary to analyse the fibrinogen degradation patterns of purified proteases from *M. xanthina* venom.

Both Na<sub>2</sub>EDTA, which is a metalloproteinase inhibitor, and the protease inhibitor cocktail containing serine, cysteine and metalloprotease inhibitors were unable to inhibit completely the fibrinogenolytic activity of *M. xanthina* venom extracts. Although some of the small bands disappeared after 120 minutes of incubation, the stability of the fragments of 27 and 31 kDa confirmed the incomplete inhibition (Fig. 3). In particular, the partial hydrolysis of the fibrinogen A $\alpha$ -chains in the presence of EDTA suggests that the responsible enzyme is a serine protease. However, inhibition of B $\beta$ -chain hydrolysis confirms that some of the fibrinogenolytic activity arises from metalloproteinases.

In the present study, the occurrence and inhibition of fibrinogenolytic activity of *M. xanthina* venom were clearly ob-

served. For further analysis, the isolation, identification, and characterization of individual venom components will provide insight into their function and biological roles.

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