

RESEARCH PAPER

Biochemical characterization of *Walterinnesia morgani* (desert black cobra) venom (Serpentes: Elapidae)

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Abstract

Snake venom contains various bioactive proteins and peptides, of which enzymes make up a significant portion. Desert Black Cobra (*Walterinnesia morgani*) is a venomous snake distributed mainly in the Middle East including southeastern Türkiye. The aim of the present study is to investigate the key enzyme activities and protein profile of *W. morgani* venom originating from Sanliurfa province. After the determination of the protein content, the venom sample was subjected to enzymatic activity assays to assess phospholipase A₂, protease, L-amino acid oxidase, hyaluronidase, 5'-nucleotidase and, phosphodiesterase activities by a spectrophotometry-based method. Protease activity was also assessed by gelatin zymography. Additionally, the fibrinolytic activity of the venom was evaluated using fibrinogen zymography and SDS-PAGE methods. The protein profile was obtained by SDS-PAGE (both reduced and non-reduced) and reversed-phase HPLC methods. According to the results, 11 protein bands between approximately 12-240 kDa were observed on non-reduced SDS-PAGE gel while there were nine bands between 12-140 kDa on the reduced gel. Venom proteins of *W. morgani* were found predominantly between 25-12 kDa. Proteins were separated into at least 19 major and minor protein groups (peaks) by HPLC analysis. The venom of *W. morgani* showed all tested enzyme activities at varying levels.

Introduction

Snake venom is a complex cocktail consisting of various bioactive proteins and peptides as major constituents, and other organic molecules and inorganics as minor components (Chippaux, 2006; İğci & Ozel Demiralp, 2020). Enzymes make up a significant portion of snake venom proteins. The main enzyme families found in snake venoms are metalloproteinase (SVMP), serine proteinase (SVSP), phospholipase A₂ (PLA₂), L-amino acid oxidase (LAO), hyaluronidase, phosphodiesterase (PDE), and 5'-nucleotidase (Chippaux, 2006). Annually, more than 420,000 venomous snakebite cases occur globally, of which about 20,000 are fatal (Kasturiratne et al., 2008). Although snake venom components are responsible for their toxicity, they also have diagnostic and therapeutic potential. The drugs targeting the cardiovascular

system such as captopril, eptifibatide, and tirofiban were developed based on the peptides found in viper venoms. Moreover, viper venom proteases are used in the diagnostic test for coagulation disorders (Von Reumont et al., 2022).

Walterinnesia morgani (Mocquard, 1905) (Desert Black Cobra), a venomous snake, is mainly encountered in the Middle East including southeastern Türkiye, Iran, Iraq, Syria, Saudi Arabia, and Kuwait (Nilson & Rastegar-Pouyani, 2007). It is the only member of the Elapidae family in Türkiye, occurring in Sanliurfa and Kilis provinces (Üçeş & Yıldız, 2020; Yıldız, 2020). Populations of *W. morgani* had known as *W. aegyptia* until it was described as a separate species (by combination) in 2007 (Nilson & Rastegar-Pouyani, 2007). It does not cause so many snakebite cases (based on the literature record) possibly due to its

cryptic nocturnal lifestyle but its bite shows neurotoxic effects such as respiratory distress, limb weakness, and numbness (Amr et al., 2020).

The venom arsenals of *W. morgani* and *W. aegyptia* were characterized by a recent proteomic study at the protein family level (Calvete et al., 2021). Three-finger toxin, PLA₂, cysteine-rich secretory protein (CRISP), Kunitz-type serine proteinase inhibitor-like protein, SVMP, LAAO, PDE, acetylcholinesterase, nerve growth factor, 5'-nucleotidase, endonucleotidase, vascular endothelial growth factor protein classes were identified in *W. morgani* venom. Moreover, some of the venom proteins (eg. PLA₂, PDE, three-finger toxins, Kunitz-type protease inhibitor) of a closely-related species *W. aegyptia* were purified and characterized previously (Abid et al., 2020; Al-Saleh et al., 2011; Duhaime et al., 1996; Lee et al., 1976; Samejima et al., 1997; Simon & Bdolah, 1980; Tsai et al., 2008). However, no study has been published until now regarding the comprehensive enzymatic and fibrinolytic activities of *W. morgani* venom. While clinical cases of Desert Black Cobra envenomation are not very common, its venom is understudied and contains different types of biologically active proteins and peptides as other snake venoms. The aim of the present study is to achieve biochemical characterization of *W. morgani* venom originating from southeastern Türkiye in terms of the key snake venom enzyme activities and protein profile.

Materials and Methods

Chemicals

All chemicals used in experiments were molecular biology or liquid chromatography grade. Bis(4-nitrophenyl) phosphate, hexadecyltrimethylammonium bromide (CTAB), tris, hyaluronic acid, fibrinogen from human plasma, phosphate-buffered saline (PBS), glycine, sodium dodecyl sulfate, calcium chloride, L-leucine, O-dianisidine dihydrochloride, and 40% acrylamide/bis solution were purchased from Sigma-Aldrich (Merck-Millipore). Bovine serum albumin (BSA) standard (2 mg/mL), horseradish peroxidase (HRP), and Bradford reagent were from Thermo Scientific, and ammonium persulfate and Coomassie brilliant blue G-250 were from AppliChem and Amresco, respectively. Triton X-100 and gelatin were purchased from Bio-Rad. Other chemicals were obtained from Merck (Merck-Millipore).

Preparation of the venom sample

Venom was milked from one adult individual of *W. morgani* collected from Keberli Village, Sanliurfa province (southeastern Anatolia) following appropriate ethical procedures as described before by Igci and Demiralp (2012). The venom sample was centrifuged at 5,000 x g speed for 5 min at 4°C and the supernatant was lyophilized by using a bench-top freeze-dryer (Millrock). Lyophilized venom was stored at -20°C until

use. The species of the snake was identified according to Nilson and Rastegar-Pouyani (2007).

Determination of the protein concentration

Venom was reconstituted in deionized water at 10 mg/mL concentration. Protein content was determined using Bradford's method (Bradford, 1976) in 96-well plate format. 10 µl BSA standards (between 200-1000 µg/mL range) and venom sample were transferred to wells, then 200 µl of Bradford reagent (Thermo Coomassie Plus) was added to each well. After 15 min incubation at room temperature, absorbance at 595 nm wavelength was measured by using a multi-plate reader spectrophotometer (Perkin Elmer, Victor3). The experiment was carried out in triplicate and mean values were used for calculations.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Discontinuous SDS-PAGE was performed using standard procedures as described by Özel Demiralp et al. (2014) using Bio-Rad Mini-Protean Tetra Cell vertical electrophoresis system. 20 µg venom sample was loaded onto gel (4% stacking, 12% running gel) in reduced (by dithiothreitol) or non-reduced conditions. Electrophoresis was carried out at 100 V constant voltage. The gel was stained with 0.12% (W/V) Coomassie Brilliant Blue G-250 using the "blue-silver" method (Candiano et al., 2004). Bio-Rad Precision Plus Unstained Protein Standard was used to assess the molecular weights of protein bands. Density graphics of gel lanes were created using GelAnalyzer 19.1 software (Lazar & Lazar, 2022).

High performance liquid chromatography (HPLC)

The crude venom of *W. morgani* was separated by the reversed-phase HPLC (RP-HPLC) method using the C18 column (Agilent Poroshell 120 SB-C18, 2.7 µm, 4.6x10 mm). After filtration through a 0.45 µm polytetrafluoroethylene membrane (Agilent), 75 µg protein was injected and chromatographic separation was performed on Agilent 1220 Infinity HPLC system equipped with a diode array detector (DAD). The chromatogram was recorded at 215 nm wavelength and processed with the aid of ChemStation software (Agilent). The reversed-phase separation was achieved using a gradient method using mobile phases A (deionized water + 0.1% TFA) and B (acetonitrile + 0.1% TFA) with a flow rate of 1 mL/min. The gradient was applied as follows: 1 min B 5%, 2-20 min B 30%, 21-50 min B 80%, 51-54 min B 5%, B 5% for 1 min.

Determination of the enzyme activities by spectrophotometry

Spectrophotometric measurements of enzyme activities were performed using Perkin Elmer Victor3 or Lambda25 instruments and appropriate blank tubes were included in each assay. Three technical replicates were included where needed.

Phospholipase A₂ (PLA₂) activity

PLA₂ activity was determined using a commercial kit according to the manufacturer's instructions (Cayman secretary PLA₂ assay kit). The colorimetric assay uses a 1,2-dithio analog of diheptanoyl phosphatidylcholine as a substrate and 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) for the detection of free thiols released after PLA₂ activity at 405 nm. Absorbance values were recorded every minute for 10 min and calculations were done using the subtracted values of consecutive time points. The results are expressed as $\mu\text{mol}(\text{substrate})/\text{min}/\text{mL}$.

Protease activity

Protease activity was determined using a commercial kit according to the manufacturer's instructions (Pierce Colorimetric Protease Assay Kit), which uses succinylated casein as a substrate. After the hydrolysis of casein by proteases, free amino-terminal groups of released peptides react with trinitrobenzene sulfonic acid (TNBSA) which produces yellowish colour. Trypsin equivalents were calculated using the standard curve.

L-amino acid oxidase (LAO) activity

LAO activity was measured according to the method published by [Bergmeyer et al. \(1983\)](#) with some modifications. The reaction mixture included 10 mM L-leucine, 0.2 mg/mL *o*-dianisidine dihydrochloride, and 100 U/mL horseradish peroxidase in 0.2 M Tris-HCl buffer (pH 7.6). Venom (50 μg) was added to this solution and absorbance was recorded every minute for 5 min. Means of the subtracted absorbance values of consecutive time points were calculated and the result is expressed as Units/mg (1 Unit = 1 μmol amino acid oxidized in 1 minute in 1 mL).

Hyaluronidase activity

Hyaluronidase activity assay was performed using the turbidimetric method ([Ferrante, 1956](#)). Reaction tubes included 50 μg hyaluronic acid and different concentrations of crude venom (between 200-6.25 $\mu\text{g}/\text{mL}$) in 0.2 M acetate buffer (pH 6.0). After 15 min incubation at 37°C, 2.5% CTAB in 2% NaOH was added to stop the reaction. Samples were measured at 400 nm. The remaining hyaluronic acid amount was estimated based on a calibration curve obtained using different concentrations of hyaluronic acid in the same conditions. After calculation, percent inhibition (hydrolyzed hyaluronic acid) values were used for the IC₅₀ calculation.

5'-nucleotidase activity

The 5'-nucleotidase activity was assayed based on a phosphate determination method by [Eibl and Lands \(1969\)](#) with some modifications. The 5'-adenosine monophosphate (5'-AMP) was used as substrate. Crude venom (50 μg) was added to the reaction mixture

containing 10 mM MgCl₂, 50 mM NaCl, 10 mM KCl, 50 mM Tris, and 10 mM 5'-AMP and incubated at 37°C for 30 min. Thereafter, 30 μl of Triton-X was added and mixed, followed by the addition of 300 μl of 2.5% ammonium molybdate solution (in 6 N H₂SO₄) and 1 mL water. After 20 min incubation at room temperature (RT), absorbance was measured at 660 nm. Released inorganic phosphate content was estimated based on a calibration curve established with KH₂PO₄. The result is expressed as μmoles of inorganic phosphate released in a minute.

Phosphodiesterase (PDE) activity

PDE activity was determined based on the method by [Trummal et al. \(2014\)](#), which is modified from the original method by [Babkina and Vasilenko \(1964\)](#). Fifty μl of venom (1 mg/mL) was added to 200 μl of 10 mM bis(4-nitrophenyl) phosphate (in 0.1 M Tris-HCl, pH 8.8) and incubated at 37°C for 30 min. After that, 1.25 mL of 0.1 M NaOH was added to stop the reaction and measured at 400 nm wavelength. Absorbance was recorded every minute for 5 min. Means of the subtracted absorbance values of consecutive time points were calculated and the result is expressed as Units (1 Unit = 1 μmol substrate modified in 1 minute in 1 mL).

Zymography

Protease activity was also determined by gelatin zymography. Gelatin was added and solubilised in the 12% resolving gel solution at a final concentration of 1 mg/mL. 25 μg of venom were mixed with SDS-PAGE loading buffer without reducing agent and electrophoresis was performed as described previously. After that, the gel was incubated in washing buffer containing 2.5% Triton X-100, 50 mM Tris-HCl pH 7.5, 50 mM CaCl₂, 1 μM ZnCl₂, and 100 mM NaCl for 2 \times 30 min at room temperature; followed by the incubation buffer (1% Triton X-100, 50 mM Tris-HCl pH 7.5, 5 mM CaCl₂, 1 μM ZnCl₂, and 100 mM NaCl) overnight at 37°C. Then it was washed with distilled water for 10 minutes, followed by staining using Coomassie Brilliant Blue G-250 for 60 min. After destaining with distilled water, the resulting gel was visualized.

Determination of the fibrinolytic activity

The fibrinolytic activity was determined according to the method by [Edgar and Prentice \(1973\)](#) using SDS-PAGE, with some modifications. Human plasma fibrinogen (12.5 μg in 5 μl) was incubated with 5 μl of venom (125 $\mu\text{g}/\text{mL}$ crude venom in 50 mM Tris-HCl, pH 7.8) for different time intervals (5, 10, 30, 60, and 120 min) at 37°C. The reaction was stopped by adding 4 μl of SDS-PAGE loading buffer, followed by heating at 95°C for 5 min. Electrophoresis was performed as mentioned before under a constant voltage of 120 V. Additionally, fibrinolytic activity was also investigated by using the fibrinogen zymogram gel method applying a similar procedure as

described in the previous section.

Results and Discussion

Venom color and protein content

The color of *W. morgani* venom used in the present study was yellowish. The yellowish color of snake venoms (typical characteristics of Viperid venoms) is generally attributed to the presence of the flavin groups on LAAOs (Chippaux, 2006). The enzymatic activity results (which are discussed below) showed that the venom had a high LAAO activity. On the contrary, Arikan et al. (2008) reported the color of *W. morgani* venom as colorless. These conflicting results may be due to the variation in the venom composition (Arikan et al., 2014; Chippaux, 2006). The protein content of the reconstituted venom sample was determined as 6.36 mg/mL by the Bradford method.

Protein profiles obtained by SDS-PAGE and HPLC

In order to get an overview of the proteome complexity of *W. morgani* venom, reduced and non-reduced SDS-PAGE and C18-RP-HPLC methods were used. The molecular weight distribution of *W. morgani* venom proteins was obtained by SDS-PAGE under reducing and non-reducing conditions. According to the results, 11 protein bands between approximately 12-240 kDa were observed on the non-reduced gel while there were nine bands between 12-140 kDa on the reduced gel (Figure 1, Table 1). High molecular weight bands of non-reduced gel at 240 and 180 kDa were not observable under reduced conditions and there were also other differences between the two gels. Venom proteins of *W. morgani* were distributed predominantly between 25-12 kDa.

Table 1. Molecular weights assigned to *W. morgani* venom protein bands according to protein marker

Band No	Non-reduced (kDa)	Reduced (kDa)
1	240	140
2	180	95
3	95	68
4	60	56
5	35	30
6	26	23
7	24	16
8	18	15
9	17	12
10	15	
11	12	

Arikan et al. (2008) separated the venom proteins of *W. morgani* from Türkiye (*W. aegyptia* name was used in the paper because it was published before the description of *W. morgani*) by polyacrylamide (7.5%) disc gel electrophoresis method and detected 12 protein fractions. Although their method does not allow the assignment of the molecular weights of the protein bands, it is observable from their results that the venom contains both lower and higher-molecular

weight proteins, corroborating the results of the present study. In a study by Abd El Aziz et al. (2015), reducing SDS-PAGE analysis of a closely related species *W. aegyptia* venom resulted in six major protein bands at 10, 12, 14, 22, 70, and 130 kDa. Two thinner and faint bands at 180 and 250 kDa were also observed. Additionally, some of the RP-HPLC fractions contained proteins around six kDa and below. Although there are shared bands between *W. aegyptia* and *W. morgani*, prominent differences are observable in SDS-PAGE band profiles. These two species are closely related to each other phylogenetically, on the other hand, they have differences in venom proteins.

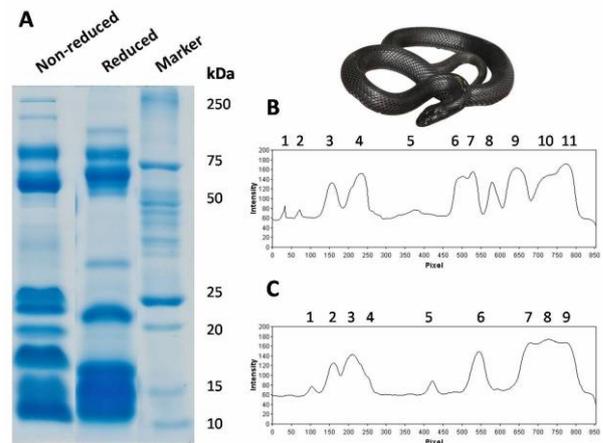


Figure 1. Protein profile of *W. morgani* venom obtained by SDS-PAGE under the reducing and non-reducing conditions. A: Gel images, B: Density plot of non-reduced gel, C: Density plot of the reduced gel. Nine protein bands were observed between 12-140 kDa on the reduced gel while 11 bands between 12-240 kDa were observed on the non-reduced gel. The gel images indicate that one of the major constituents of *W. morgani* venom is three-finger toxins and reveal the presence of high-molecular weight proteins.

Venom proteomes of *W. morgani* and *W. aegyptia* were characterized at the family level by using bottom-up and top-down proteomics in combination (Calvete et al., 2021). Three-finger toxin (3FTx) was the most abundant protein family (~40% of total venom proteins) in *W. morgani* venom. The second most abundant family was PLA₂ (26%) and cysteine-rich secretory protein (CRISP) was the third. Other families identified in *W. morgani* venom were as follows: Kunitz-type serine proteinase inhibitor-like protein (7.2%), SVMP (7.1%), LAAO (6.4%), PDE, acetylcholinesterase, nerve growth factor, 5'-nucleotidase, endonucleotidase, vascular endothelial growth factor. The SDS-PAGE image in Figure 1 shows that most of the venom proteins of *W. morgani* are under 25 kDa. Calvete et al. (2021) identified 3FTx from SDS-PAGE bands of reduced *W. morgani* venom at 10, 14, 18, and 20 kDa. A similar band pattern indicates that the venom sample used in the present study also contains 3FTx as a major venom constituent. Both the SDS-PAGE band pattern and enzyme activity results correlate well with the identified protein families from *W. morgani* venom.

Homodimeric PLA₂ group-I enzyme (named as

WaPLA₂) with an estimated molecular mass of 30 kDa was purified from *W. aegyptia* with bactericidal activities (Bacha et al., 2018). PLA₂ was identified in *W. morgani* venom from a reduced SDS-PAGE band at approximately 16 and 22 kDa molecular weight and from a 31 kDa band under non-reducing conditions (Calvete et al., 2021). A similar band pattern was observed in this study indicating the presence of dimeric PLA₂ enzymes in *W. morgani* venom, and also it was found as one of the major venom constituents according to the band intensities.

The molecular weight of a single-chain phosphodiesterase-I purified from *W. aegyptia* venom was estimated as 158 kDa (Al-Saleh & Khan, 2011). PDEs found in snake venoms generally have higher molecular weights between 90-150 kDa (Al-Saleh & Khan, 2011; Dhananjaya et al., 2010). Although most of the snake venom PDEs are monomeric, some of them were reported as homodimers (Al-Saleh & Khan, 2011). The SDS-PAGE results of the present study showed that *W. morgani* venom has three bands at 95, 180, and 240 kDa under non-reducing conditions whereas 95 and 140-kDa bands were observable in reduced wells in the range where most snake venom PDEs are found (Figure 1). From the results, it is possible that *W. morgani* venom contains single-chain ~ 95 kDa PDE, which is different from *W. aegyptia*. Supporting the results, Calvete et al. (2021) also identified PDE from a reduced SDS-PAGE band with about 97 kDa in *W. morgani* venom.

Based on the enzyme activity results (see the next section) as well as the known molecular weights of SVMPs from other snake venoms, the 240 kDa-band observable on non-reducing SDS-PAGE gel indicating that *W. morgani* venom contains high molecular weight multimeric P-III group SVMP. Calvete et al. (2021) identified SVMP from a non-reduced SDS-PAGE band above 116 kDa and from reduced bands, as well. The present study provides the more approximate molecular weight of the multimeric SVMP found in the venom of *W. morgani*. A ~60 kDa-serine proteinase with factor X activating activity was also purified from *W. aegyptia* venom (Khan & Al-Saleh, 2015). A band at approximately 60 kDa is present in the non-reduced SDS-PAGE gel of *W. morgani* venom, indicating the possibility of the presence of a serine proteinase.

Venoms of the snakes belonging to the family Elapidae may have predominantly proteins below 20 kDa (especially *Naja*, *Bungarus*, *Ophiophagus*) (Nawarak et al., 2003). However, proteins with higher molecular weights can also be found as a major component in the venoms of other Elapids (eg., *Notechis*, *Pseudonaja*, *Oxyuranus*) (Birrell et al., 2007). The presence of the proteins with higher molecular weight in the venoms of *W. morgani* and *W. aegyptia* observed in the present study and the others is a prominent result in this regard, showing the unique venom composition of the genus (Abd El Aziz et al., 2015; Calvete et al., 2021). The genus *Walterinnesia* is

considered and used as a basal clade in the phylogenetic trees of Elapids and is recognized as one of the most primitive Elapid species (Keogh, 1998; Wüster et al., 2007). Therefore, investigations on the venom of this genus may provide novel data regarding the venom evolution.

W. morgani envenomation causes generalized limb weakness, numbness, respiratory distress, paraesthesia around the wound, and diplopia, related to its neurotoxic effect (Haidar & Deitch, 2015; Lauer et al., 2011). Edema around the wound can also be seen. These effects mainly originate from the abundant 3FTx proteins in *W. morgani* venom. But other secondary protein families such as PLA₂, SVMP, and LAAO also contribute to its physiological effects.

According to the HPLC result recorded at 215 nm, *W. morgani* venom proteins separated into 19 protein groups (peaks) between 15-51 min (Figure 2), which corresponds to 20%-80% acetonitrile concentration in our method. But only two minor peaks were recorded after 60% acetonitrile.

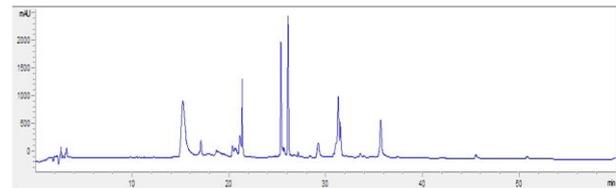


Figure 2. HPLC chromatogram of *W. morgani* venom measured at 215 nm wavelength.

The chromatogram profile obtained at 280 nm was similar, therefore it is not shown. The retention times of each peak are indicated in Table 2. Major and minor peaks were observable on the chromatogram.

Table 2. Retention times of the major and minor RP-HPLC peaks of *W. morgani* venom

Peak No	Retention time (min)	Peak No	Retention time (min)
1	15.2	11*	27.1
2	17.1	12*	28.3
3*	18.7	13	29.2
4	20.3	14	31.2
5*	20.6	15	31.5
6	21.1	16*	33.5
7	21.3	17	35.6
8	25.3	18*	45.5
9	25.7*	19*	50.8
10	26.1		

The general pattern and peak clusters of the chromatogram show similarity to those provided by Calvete et al. (2021). Although chromatographic conditions are different, correlating the retention times and acetonitrile concentration allows a certain degree of comparison. For instance, four region of peak groups is observable on the chromatogram provided by Calvete et al (2021) which is consistent with our results. With a rough estimate, HPLC peaks between 14-22 min obtained in the present study may contain 3FTx, PLA₂

may be found in the peaks between 25-27 min, CRISP and SVMP between 30-32, LAAO between 35-37 min retention times. But of course, precise identification can be achieved by mass spectrometry analysis. Both SDS-PAGE and HPLC results indicate that *W. morgani* venom sample investigated in the present study is rich in 3FTx and PLA₂.

Enzyme activities

Enzymatic activities were measured based on spectrophotometric methods. Additionally, protease activity was assessed by gelatin zymography. The results of the enzyme activity measurements by spectrophotometry are summarized in Table 3. Results are generally presented as $\mu\text{mol}/\text{min}/\text{mL}$ (U/mL). The protein concentration of the venom was calculated as 6.36 mg/mL by Bradford's method and can be used for U/mg calculations. While there is very limited information on the venom of *W. morgani*, there are a few studies on *W. aegyptia* venom, a closely related species aiming to purify and characterize venom proteins. Therefore, the results are compared with those related to *W. aegyptia* venom when available.

Table 3. Enzyme activities of *W. morgani* venom

Enzyme Activity	Activity Values
Phospholipase A ₂	68.8 U/ml
Protease (spectrophotometric)	–
Protease (gelatin zymography)	Thin clear band at about 240 kDa
L-amino acid oxidase	4.4 U/ml
Hyaluronidase	IC ₅₀ = 27.45 $\mu\text{g}/\text{ml}$
Phosphodiesterase	2.93 U/ml
5'-nucleotidase	82.66 U/ml

According to the results of the present study, *W. morgani* venom showed well PLA₂, LAAO, PDE, hyaluronidase, and 5'-nucleotidase activities. These results are similar to those reported by Al-Asmari et al. (1997) obtained for *W. aegyptia* venom. Protease activity was not detected spectrophotometrically in our experimental conditions but a thin clear band at about 240 kDa was observed on gelatin zymogram gel (Figure 3B). There is also a thin band at the same kDa on the non-reducing SDS-PAGE gel, indicating that *W. morgani* venom may contain high molecular weight (approximately 240 kDa) multimeric SVMP. The reason for not detecting the activity in a spectrophotometry-based method can be related to the sensitivity of the assay (the positive control showed activity) or substrate specificity. Gelatin was used as a substrate in zymography whereas casein was used in the spectrophotometric method. Zinc-dependent metalloproteinases (EC 3.4.24) in snake venoms play an important role in the pathology of a venomous snakebite by interfering with the coagulation cascade and causing tissue necrosis and hemorrhage (Chippaux, 2006). SVMPs are mainly found in viper venoms as a major venom protein family. It was concluded that

although *W. morgani* venom includes SVMP, its activity is low, which is also the case in the *W. aegyptia* venom (Al-Asmari et al., 1997).

PLA₂ (E.C. 3.1.1.4) is one of the common protein families found in snake venoms that hydrolyses the sn-2 ester bond of glycerophospholipids resulting in the generation of fatty acids and lysophospholipids. Besides their action on the tissue damage in the prey, they have diverse biological activities including procoagulant, anticoagulant, antiplatelet, antimicrobial, myotoxic, and neurotoxic effects (Chippaux, 2006; Kini, 2003). PLA₂ enzymes were purified from the venom of *W. aegyptia* previously (Abid et al., 2020; Bacha et al., 2018; Simon & Bdolah, 1980). PLA₂ activity of the crude venom was calculated as 750 U/mg total protein and about four-fold purification was achieved in the study by Simon & Bdolah (1980). The calculated activity unit value is much higher than that obtained in the present study. The reason for this can be technical variation, different methods were used in these two studies. For example, the specific activity of PLA₂ was calculated as 50 U/mg again for *W. aegyptia* venom. In another study that used a method more similar to the one in this paper, lower activities close to those calculated in this study (when converted to the same unit) were reported across different *Naja* species (Tan et al., 2019). Moreover, enzyme amounts/activities may vary between different species (Chippaux, 2006). Even though they are closely related, it was shown that enzyme abundancies (including PLA₂) varied significantly between *W. aegyptia* and *W. morgani* (Calvete et al., 2021).

LAAO (E.C. 1.4.3.2) is another enzyme family found in snake venoms commonly. *W. morgani* venom has a good level of LAAO activity according to this study. Calvete et al. (2021) identified the enzyme from different SDS-PAGE bands, which are consistent with the results of the present study. LAAOs, which catalyze the oxidative deamination of L-amino acids, have various activities such as hemorrhagic, anticoagulant, antiplatelet, apoptosis-inducing, antibacterial and antiviral effects (Du & Clemetson, 2002). Substrate specificity of *W. morgani* LAAO can be assessed using amino acids other than leucine by additional studies.

Phosphodiesterases (E.C. 3.1.4.1) cleave phosphodiester bonds in nucleic acids and hydrolyze some type of nucleotides, as well. They show diverse biological activities related to nucleotide signaling including platelet aggregation (Chippaux, 2006; Trummal et al., 2014). Phosphodiesterase-I was purified from the venom of *W. aegyptia* (the locality is not clear but published after the description of *W. morgani* so that the taxon was considered as *W. aegyptia*) using preparative native polyacrylamide gel electrophoresis (Al-Saleh & Khan, 2011). They found that the enzyme had a specific activity of 3.4 U/mg (this value corresponded to a three-fold purification). PDE

enzymes were purified from various Viperids and Elapids, as well but Viperids have generally higher activity (Dhananjaya et al., 2010). Along with snake venom PDEs, 5'-nucleotidase (E.C. 3.1.3.5.) is another understudied enzyme class found in snake venoms that cleaves variety of mononucleotides. These enzymes prefer 5'-AMP as a substrate. Therefore, this molecule was used as a substrate to assess the enzymatic activity. Releasing of adenosine by the 5'-nucleotidase activity is thought to contribute to the toxicity of snake venoms (Dhananjaya et al., 2010).

Hyaluronidase (E.C. 3.2.1.35) is another common enzyme family of snake venoms (even across other venomous animal groups). Although its abundance is generally lower than other snake venom proteins, they show significant activity by degrading mucopolysaccharides in the connective tissue of the prey. Therefore, it is thought that this enzyme "helps" venom diffuse through the tissues (Chippaux, 2006). Although hyaluronidase activity of *W. aegyptia* venom was reported (Al-Asmari et al., 1997), there is no information regarding the presence of this enzyme in *W. morgani* venom in the literature. Venom samples were obtained from different parts of Riyadh (Saudi Arabia) in the aforementioned study before the description of *W. morgani*; both species were recorded from the region and specimens identified as *W. morgani* were found genetically identical to *W. aegyptia* from Riyadh and Egypt (Alshammari et al., 2022). Therefore, the species used for the venom source was considered as *W. aegyptia*. Calvete et al. (2021) did not identify the enzyme either by bottom-up or top-down proteomic analyses, possibly due to the low abundance.

In a recent study, proteomic characterization of *W. morgani* venom was achieved and all the enzyme classes assayed in the present study except hyaluronidase were identified at the protein family level (Calvete et al., 2021). As discussed above, SDS-PAGE results also confirm the presence of these enzyme groups in the venom. This study provided the first data on the activities of these enzyme groups in *W. morgani* venom. Additionally, the presence of the enzyme hyaluronidase was reported in *W. morgani* venom for the first time in the present study.

The fibrinogenolytic activity was also assessed by SDS-PAGE and fibrinogen zymography. A slight activity was observed in gelatin zymogram gel while no activity was detected in fibrinogen zymography (Figure 3C). Moreover, SDS-PAGE analysis shows no significant degradation of fibrinogen chains by *W. morgani* venom proteases. Fibrinogen degradation is especially important in the envenomation cases of Viperids (Chippaux, 2006). Although the results of the present study and those in the literature indicate the presence of SVMP and possibly SVSP in *W. morgani* venom, these enzymes do not have (at least prominent) fibrinogenolytic activities according to the results.

Supporting these results, lacking the defibrinogenating activity of *W. aegyptia* (a closely related species) was also reported by Al-Asmari et al (1997).

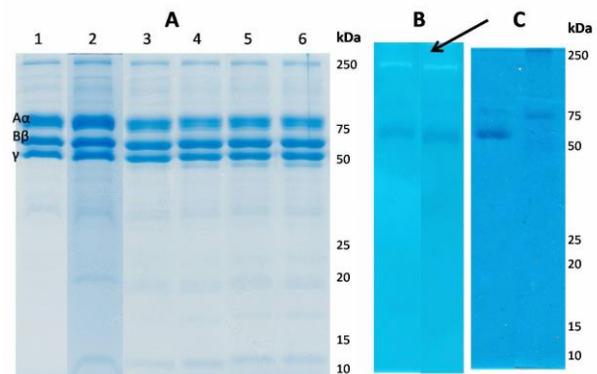


Figure 3. Gel images of SDS-PAGE based fibrinogenolytic activity (A), gelatin zymography (B) and fibrinogen zymography (C). Arrow indicates the slight gelatinolytic activity of *W. morgani* venom. Lane 1: untreated fibrinogen; lanes 2-6: fibrinogen incubated with venom at 5, 10, 30, 60, 120 min, respectively. A α , B β , and γ are symbols denoting the alpha, beta and gamma chains of fibrinogen protein, respectively.

Briefly, the present study revealed the key enzymatic activities of *W. morgani* venom in a comparative manner as well as reported hyaluronidase for the first time and provided additional information on its protein profile. Published studies have been generally focused on the venom of a closely related species *W. aegyptia*. Hence, the data on *W. morgani* venom is limited. Moreover, its fibrinogenolytic activity was assessed for the first time in the literature with this study. There is no data regarding the bioactivity of *W. morgani* in the literature to the best of the author's knowledge. However, a closely related species *W. aegyptia* was shown to possess anticancer and antimicrobial activities (Al-Sadoon et al., 2012; Bacha et al., 2018; Badr et al., 2013). Some interesting molecules such as a peptide named actiflagelin (a member of the 3FTx family), which has sperm motility-enhancing activity have been isolated from *W. aegyptia* venom (Abd El-Aziz et al., 2018). Taking into consideration these findings, *W. morgani* venom may also have potential as a source for natural bioactive molecules.

Conclusion

In conclusion, *W. morgani* venom was found to have all the major enzyme activities important for snake venoms and its venom contains both high and low-molecular weight proteins. The findings of the present study highlight *W. morgani* venom as a good candidate for use in the discovery of novel bioactive proteins. Analyzing snake venoms in order to obtain an inventory of the protein/toxin classes and assess the variation is not only important for biodiscovery, but also provides insight into the envenomation pathology and contributes to antivenom production. The results are useful in this regard and will guide future purification and characterization studies.

Ethical Statement

Venom was extracted from one adult individual of *W. morgani* collected from Keberli Village, Sanliurfa province (southeastern Anatolia) following appropriate ethical procedures. Although collecting venom in the nature does not require ethical permission since it can be considered as a specialized secretion, the author obtained ethical permission for animal handling in the laboratory and care as well as the venom milking procedure for various species from Ege University Animal Experiments Local Ethics Committee (2010-43). This study was presented in 1st International Congress on Biotech Studies.

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