VNU Journal of Science: Natural Sciences and Technology, Vol. 41, No. 2 (2025) 71-78

VNU Journal of Science: Natural Sciences and Technology

Journal homepage: https://js.vnu.edu.vn/NST

Original Article

Investigation of the Venom Protein Profile of the Malayan Pit Viper *Calloselasma rhodostoma* (Kuhl, 1824) from Vietnam and Toward Toxin Neutralization

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Received 15th September 2024
Revised 22nd April 2025; Accepted 06th May 2025

Abstract: Nearly 60 snake species belonging mainly to the Viperidae and Elapidae families are critical threats to human health in Vietnam with thousands of snakebite victims annually. *Calloselasma rhodostoma* − the Malayan pit viper is documented to cause high mortality rates in Southeast Asia. The major components of snake venom include SVMPs (Snake Venom Metalloproteinases), SVSPs (Snake Venom Serine proteases), and PLA_2 (Phospholipase A_2). Information regarding the venom protein composition plays a crucial role in antivenom compound research and development. However, currently, there is no study carried out to investigate these major toxins and their activities in the Malayan pit viper in Vietnam. Using consecutive differential centrifugation, sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, and plate diffusion assay, we demonstrated the presence of several proteases in the venom of *C. rhodostoma* from Vietnam. In the supernatant fractions, five dominant bands predicted to represent groups of SVMPs, PLA_2, SVSPs could be observed. Three concentrated fractions, C100, C50, and C10 (filtered by 100−kDa, 50−kDa and 10−kDa column, respectively) by showed proteolytic activity with the largest substrate clear zone belonging to the fraction containing higher molecular weight SVMPs. This study provided for the first time evidence of venom toxins' presence in the venom of the Malayan pit viper from Vietnam and their activity towards the screening of antivenom drugs.

Keywords: Diffusion assay, *Calloselasma rhodostoma*, proteolytic activity, snake venom proteins, snakebite.

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https://doi.org/10.25073/2588-1140/vnunst.5826

1. Introduction

Vietnam experiences a tropical monsoon climate [1], and is known for its remarkable biodiversity. Animal species are spread across its plains, midlands, and mountainous areas [2], contributing to its rich variety of life. These natural features provide an ideal environment for the growth and proliferation of various animal species, including snakes. The venomous snake can be classified according to the physiological effects of their venom into three types: neurotoxic, hematotoxic, and myotoxic [3]. In Vietnam, there are approximately 260 snake species belonging to 10 distinct families, of which about 60 species have been identified as venomous and potentially dangerous to humans, with some even capable of causing death [4].

Calloselasma rhodostoma - the Malayan Pit Viper (family Viperidae, subfamily Crotalinae), is a species that is notably responsible for severe morbidity, disability, and mortality across Cambodia, the Indonesian islands of Java and Madura, Peninsular Malaysia, Myanmar, and Vietnam [5-7]. This snake species is predominantly found in Vietnam's central and southern regions, spanning from Gia Lai to An Giang [8]. Due to its wide distribution, large population, and natural habitat overlapping with areas where people work, such as farms, fields, and industrial plantations, there have been numerous recorded cases of bites caused by the Malayan pit viper. Data obtained from 2011 to 2013 in Ho Chi Minh City, Dong Nai Province, and Ba Ria-Vung Tau Provinces indicates that Malayan pit viperbites comprised 11.3%, 12%, and 56% of the total cases reported in these regions, respectively [9]. Patients typically experience severe pain, swelling, and bleeding at the site of the bite, along with the formation of numerous blisters. In 77.5% of cases, bleeding from the wound was documented, and 62.5% of cases involved blistering of the affected limbs. Bleeding from ruptured blisters was particularly dangerous and could occasionally prove fatal. Intense, immediate pain was reported in 70% of cases, while necrosis at the bite site occurred in 17.5% of cases [9].

The venom of the *C. rhodostoma* is a complex mixture of various proteins, each playing a distinct role in the snake's toxic effects. Some notable protein families in the venom of the Malayan pit viperinclude SVMPs (Snake Venom Metalloproteinases - 61.31%), SVSPs (Snake Venom Serine proteinases - 11.58 %), PLA_2 (Phospholipase A2 - 7.39%) and other protein families (1.77%) [10]. Each major protein family is further divided into different groups, *e.g.* SVMP groups P − I to P − III or 14 groups for PLA_2 [11]. The groups within an individual family could vary substantially in sequences and molecular weights, yet share common catalyzed reactions. Thus, conventional methods used to investigate the snake venom proteins often include SDS-PAGE electrophoresis and relevant activity assay (*i.e.* caseinolytic assay for proteases, hemolysis assay for PLA_2) [12, 13]. In Vietnam, there was only preliminary research on snake venom proteins, focusing mostly on protein separation [14, 15]. This study provided for the first time evidence for the relative abundance of the major proteins in the venom of the Malayan pit viper (*C. rhodostoma*) from Vietnam together with the demonstration of the protease activity from the obtained protein fractions.

2. Experimental

2.1. Sample Collection and Identification

Ethical approval for animal research was received under code 25-2023/NCHG. The snake specimens were collected during field surveys in provinces where the *C. rhodostoma* is known to occur, using specialized snake hooks for capture. Specimens were deposited in the collections of the Institute of Genome Research (IGR), Vietnam Academy of Science and Technology (VAST), Hanoi, Vietnam.

The taxonomic identification was carefully performed based on the authoritative references by Kadafi et al., [16]. Additionally, relevant scholarly articles are referenced in the species

descriptions. The common names and geographic distributions strictly adhere to the classifications provided by U. Peter [4] and N. V. Sang et al., [8].

Morphological characters: All measurements were meticulously taken from preserved specimens using a slide caliper with a precision of 0.1 mm except for body and tail lengths. Measurements of the body were rounded to the nearest millimeter. The number of dorsal scale rows was assessed at three specific locations: one head length posterior to the head, at mid-body, and one head length anterior to the vent. Scalation characteristics were examined using a binocular microscope.

The abbreviations used for the morphometric measurements are as follows: **Ven:** Ventral plates; **Sc:** Subcaudal plates; **Cloacal:** Single/divided; **Sup (r/l):** Number of supralabials on the right/left; **PreOc (r/l):** Number of preoculars on the right/left; **Subocular:** Present/absent; **PostOC (r/l):** Number of postoculars on the right/left; **Inf (r/l):** Number of infralabials on the right/left.

2.2. Venom Collection

The snake's mouth was thoroughly cleaned, and the snake was then induced to bite a glass container sealed with a parafilm membrane. The snakes selected for this procedure were mature individuals, approximately one meter in length, and had fasted for at least 10 days. Once the snake had firmly latched onto the container, the venom glands at the corners of its mouth were gently massaged and pressed using the right thumb and forefinger, allowing the venom to flow through its fangs into the glass gradually. This method enables the snake to resume normal feeding habits afterward, as its fangs do not come into contact with hard objects, preventing injury. After extraction, the venom was centrifuged at 4 °C at 5,000 rpm for 10 minutes. The protein content of all venom samples was measured using the Bradford assay by Bradford (1976) [17], with an initial concentration of 380.71 mg/mL.

2.3. Preparation of C. rhodostoma Venom Fractions and SDS-PAGE Analysis

The crude venom was first fractionated into soluble and insoluble fractions. Following centrifugation at 10,000 rpm to remove any debris, the clear supernatant was diluted 1:50 with PBS buffer, and then passed through a 100-kDa MWCO centrifugal filter unit (to concentrate proteins with molecular weights of 100 kDa or higher. Subsequently, the flow-through was processed using a 50-kDa MWCO centrifugal filter unit (second concentrator), and the resulting flow through was applied on the third concentrator, a 10-kDa MWCO centrifugal filter unit. The separated fractions were then analyzed using a 12% polyacrylamide gel.

2.4. Determination of the Relative Intensity of the Bands from the Electrophoresis Gel

ImageJ densitogram suite was used to estimate the band intensity after photographing the SDS-PAGE gel. Briefly, each lane was selected and compared with the others, generating a plot, in which, the intensities were displayed as peaks. The peak areas corresponding to the bands were measured with the embedded tool and the ratios were calculated using Microsoft Excel. Each band was measured and calibrated using the straight line function integrated in ImageJ suite.

2.5. Casein Agar Plate Assay

The assay was performed based on the method described by Yuan and Cole (1987) [18], with a few modifications: 0.5 g of agar was dissolved in 46 ml of 0.5 M Tris buffer (pH 6.5), then boiled and cooled to approximately 50 °C. Afterward, 4 ml of casein solution (0.5% in 1.0 M NaOH) was added. The mixture was poured into plates and allowed to set. Wells were then made in the solidified agar and filled with trypsin (200 µg/ml) for positive controls and the obtained fractions, which were diluted 50 times. A volume of 25 µL was used in each well, with a final concentration of 190.3 µg. The plates were incubated overnight at 37 °C. Following

the addition of 3 ml of acetic acid, distinct clear zones around the wells emerged, indicating the presence of proteolytic which was measured in triplicates to calculate the average values of the zone diameters, https://eleif.net/photomeasure.

3. Result and Discussion

3.1. Morphology Identification

Calloselasma rhodostoma (Kuhl, 1824).
English name: Malayan pit viper.
Synonym:
Trigonocephalus rhodostoma Kuhl 1824.
Agkistrodon rhodostoma De Rooij 1917.
Agkistrodon annamensis Bourret 1936.
Calloselasma rhodostoma Welch 1994.

Identification: Dorsal ground coloration varies from brown or brick-red to pinkish or yellowish; brown or black triangular spots edged with white along the dorsal mid-line. The head brown with pale stripe above eyes, from snout to occiput. Ventral is creamy with dark mottling. Iris is gray/beige/yellow.

Figure 1. *C. rhodostoma*/Malayan pit viper. Photo by Tao Nguyen Thien.

Description: The morphological characteristics of the specimen agreed well with the description of Kadafi et al., [16]. Body rather stout, cylindrical. Dorsal scale rows 22 – 21 − 17 (♂), 22 – 23 − 24 (♀), smooth; ventral: 141 − 152 (♂), 148 − 156 (♀); subcaudal: 48 − 56 (♂) and 31 − 43 (♀), respectively, entire cloacal. Head large, triangular shape, indistinct from neck, with pointed, slightly upturned snout. Eye small, straight pupils; distinct heat−sensor pit between nostril and eye. Two preoculars; subocular absent; two postocular; supralabials 7/8 (♂), 7 – 9 (♀); infralabials 10 − 12 (♂), 11 − 13 (♀).

Distribution in Vietnam [8]:

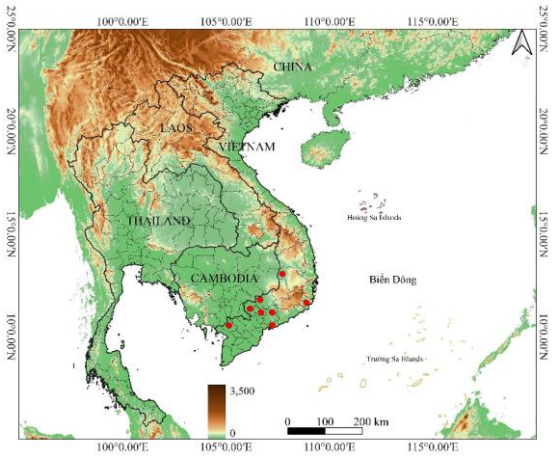


Figure 2. The distribution range of *C. rhodostoma* (Red circle) in Vietnam.

In Viet Nam , this species is found in Gia Lai, Ninh Thuan, Binh Phuoc, Binh Duong, Ba Ria–Vung Tau, Dong Nai, Tay Ninh, An Giang [8]. The distribution of *C. rhodostoma* in Vietnam offers critical ecological context for this research while underscoring the importance of investigating the venom of this species. Its venom protein profile is pivotal in unraveling toxicological mechanisms and advancing the development of effective antivenom therapies. Furthermore, this study facilitates the discovery of antivenom drugs, playing a vital role in safeguarding public health in regions impacted by snakebites.

3.2. SDS-PAGE Analysis

The electrophoresis gel (Figure 3) and the corresponding densitogram (Figure 4) showed substantial differences in both band pattern and density among the concentrated fractions. The majority of the venom proteins migrated below the 45 kDa band of the marker. Despite the unexpected separation, *i.e.* according to the theoretical molecular weight cut−offs, the C100 did retain the higher molecular weight proteins

(above 25 kDa). The unexpected separation could result from leakages of the filter membranes that allowed the molecules with weights higher than the cut-off values to flow through. These leakages have been attributed to membrane fouling that may lead to both blockages and cracks, altering the permeability of the filters [19]. There was almost a single band below the 18.4 kDa band in the C10 fraction, predicted to be PLA_2, consistent with previously published studies on the size (13−15 kDa) and relative abundance of this toxin (10−14%) in the Malayan pit viper [20, 21]. The three groups of SVMPs, P−I (kistomin), P−II (rhodostoxin), and P−III, with molecular weight ranges of 20−30, 30−60, 60−100 kDa and ratios up to 20% each could also be preliminary identified based on the band position and intensity [22].

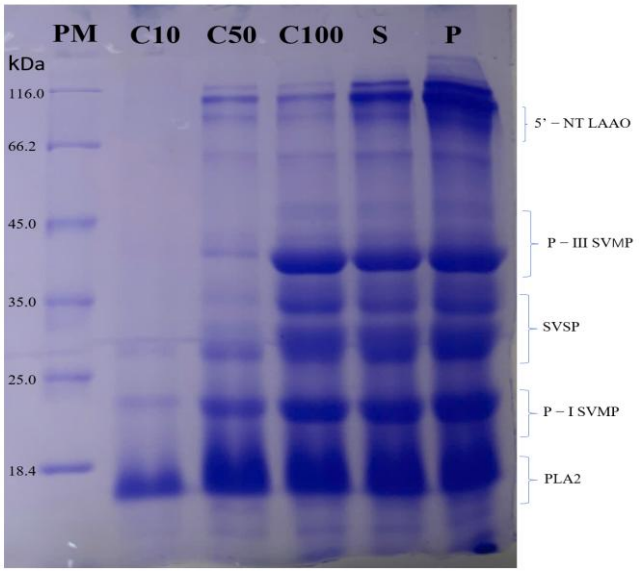


Figure 3. SDS-PAGE and predicted protein profile of *C. rhodostoma* venom fractions. PM: Thermo Sciencetific Unstained Protein MW Marker; C10: concentrated fraction using the 10−kDa filter column: C50: concentrated fraction using the 50−kDa filter column; C100: concentrated fraction using the 100−kDa filter column; S: supernatant fraction of the *C. rhodostoma* venom; P: pellet fraction of the *C. rhodostoma* venom.

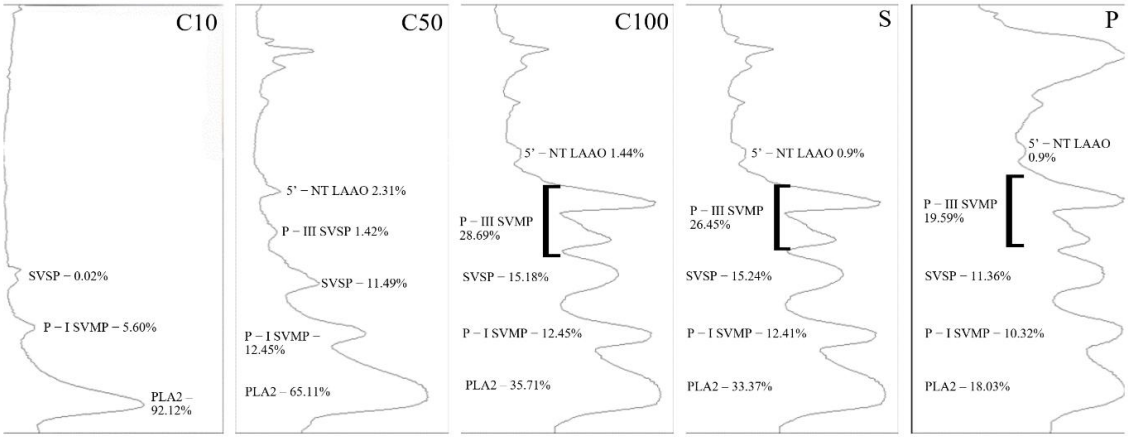


Figure 4. Densitogram of the fractions in the SDS-PAGE.

Five main protein families were identified: PLA_2, P − I SVMP, SVSP, P − III SVMP, and 5' − NT LAAO. Similar band patterns were observed for S, P, and C100 fractions that included five main peaks (Figure 4, panels C100, S, P), except the presence of certain proteins migrating above the 66.2 kDa band in the P sample. PLA_2 was the protein with the most efficiently concentrated, demonstrated by a nearly three-fold increase in ratio (from 33.37% to 92.12%). Conclusively, the differential centrifugation method could be used to enrich the venom proteins for further experiments such as size exclusion chromatography or mass spectrometry. It is important to note that identifying protein composition solely based on electrophoretic migration, without appropriate controls, may be inaccurate, as different proteins can migrate similarly. The activity assays provided another evidence for the presence of the enzyme families, however, further experiments are necessary (e.g., zymography technique that allows in-gel substrate cleavage, or column chromatography methods for protein purification before assaying [23].

3.3. Casein Agar Plate Assay

Based on Figure 6, a statistically significant difference in halo diameter was observed between C10 and C100, as well as between C50 and C100 ($p < 0.05$). The caseinolytic assay clearly demonstrated the protease activity of the snake venom proteins in all five fractions. Consistent with the electrophoresis results, the S sample (32.33 ± 0.31 mm), which exhibited less intense bands, showed a slightly smaller clear zone compared to the insoluble pellet fraction (33.37 ± 0.12 mm) (Figure 5, wells S, P). The largest clear zone was observed in the C100 sample (34.85 ± 0.32 mm), suggesting a higher concentration of proteases in this fraction. Notably, substrates other than casein can also be used to assess proteolytic activity, providing flexibility in evaluating different enzymatic properties [23, 24]. Proper substrate selection is crucial for applying this simple plate assay format to screen for venom-neutralizing compounds based on the inhibition of the highly abundant venom proteases [25].

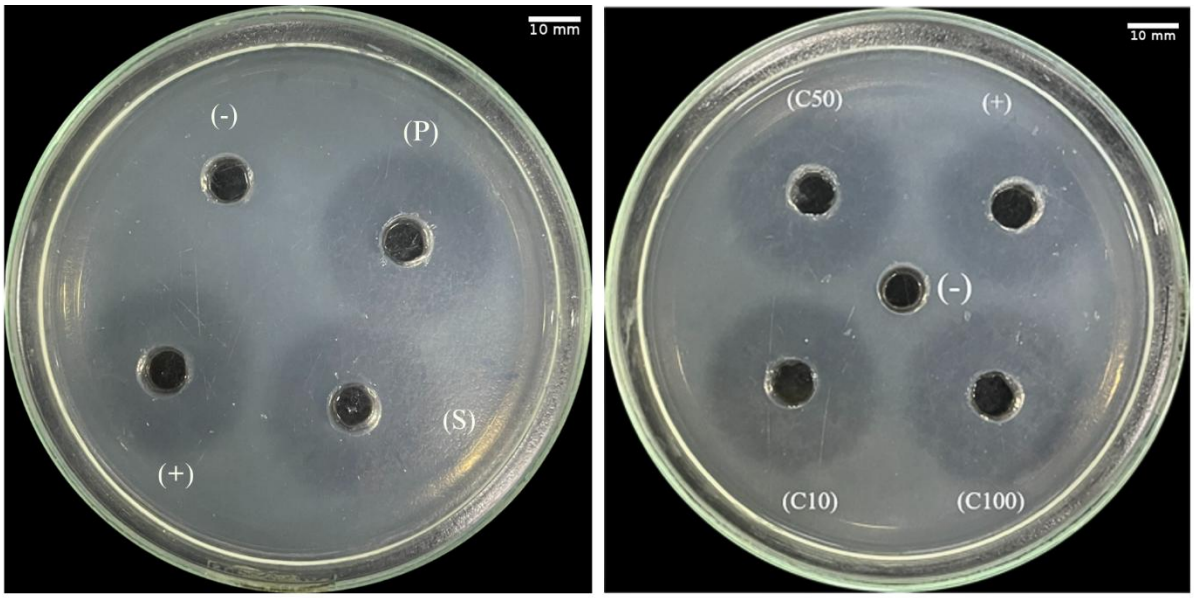
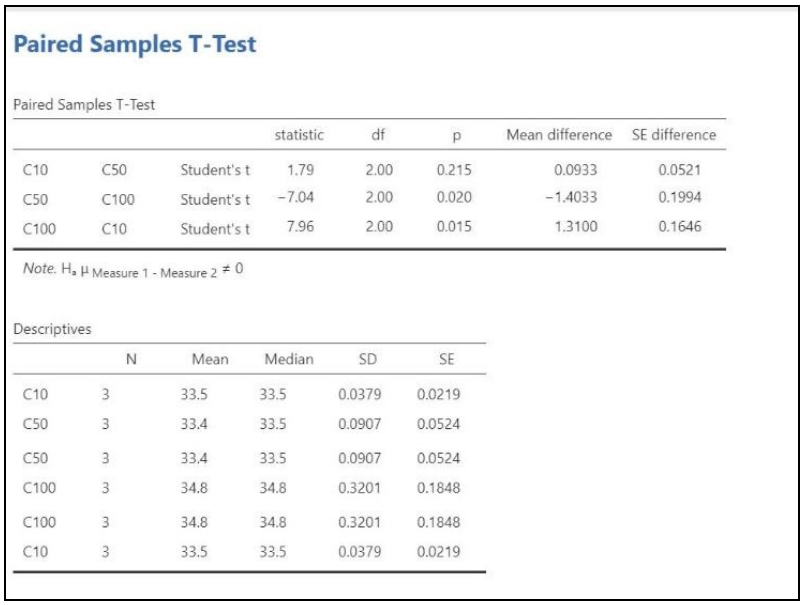


Figure 5. Caseinolytic assay results of *C. rhodostoma* venom fractions. (-): negative control (PBS); (+): positive control (trypsin 0.25 µg); (S): supernatant fraction of the *C. rhodostoma* venom ; (P): pellet fraction of the *C. rhodostoma* venom; (C100): concentrated fraction using the 100−kDa filter column; (C50): concentrated fraction using the 50-kDa filter column; (C10): concentrated fraction using the 10−kDa filter column.



Paired Samples T-Test

Paired Samples T-Test

			statistic	df	p	Mean difference	SE difference
C10	C50	Student's t	1.79	2.00	0.215	0.0933	0.0521
C50	C100	Student's t	−7.04	2.00	0.020	−1.4033	0.1994
C100	C10	Student's t	7.96	2.00	0.015	1.3100	0.1646

Note. H_a $\mu_{\text{Measure 1 - Measure 2}} \neq 0$

Descriptives

	N	Mean	Median	SD	SE
C10	3	33.5	33.5	0.0379	0.0219
C50	3	33.4	33.5	0.0907	0.0524
C50	3	33.4	33.5	0.0907	0.0524
C100	3	34.8	34.8	0.3201	0.1848
C100	3	34.8	34.8	0.3201	0.1848
C10	3	33.5	33.5	0.0379	0.0219

Figure 6. The paired sample T-test for multiple comparisions zone of inhibition between different fractions of *C. rhodostoma* venom.

4. Conclusion

Understanding the composition of snake venom proteomes is crucial for developing neutralizing therapies to address the significant number of snakebite victims worldwide, including in Vietnam. Using centrifugal columns with various molecular weight cutoffs, we concentrated dominant proteins ranging from under 18 kDa up to 116 kDa and demonstrated proteolytic activity that corresponded to that of snake proteinases. This study represents the first exploration of the venom of the Malayan pit viper from Vietnam, indicating the possible presence of highly abundant SVMPs and SVSPs. For future research, single-protein purification and mass spectrometry identification could be applied to further investigate the venom components of this species.

Acknowledgements

The authors would like to thank research grants under the 2023-2024 Joint WHO Western Pacific Region for facilitating collaboration to make this research possible. This research is supported by the WHO Small Grants 2023.

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