Original Article

Biochemistry of the Thrombin-Like Enzyme and Its Purification from Iranian Echis Carinatus Snake Venom: Its Interaction with Platelet Receptors

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ABSTRACT

Snake venoms are rich in valuable substances that have medical potential in the diagnosis and treatment of hemostatic diseases. The present paper was aimed at the purification and functional characterization basis of a thrombin-like enzyme and its role in the functioning of the coagulation cascade and platelet aggregation pathway. A thrombin-like serine protease was purified from the Iranian *Echis carinatus* venom (TLIECV), employing a one-step chromatographic procedure. This peptide was collected in high yield and purity by a single chromatographic step using RP-HPLC equipped with a C_{18} column. This peptide showed a 3000 Da molecular weight in gel-electrophoresis. Evidence in the SDS-PAGE gel has confirmed high recovery of fraction in optimal terms. Subsequently, this peptide was identified via its intact molecular mass and peptide mass fingerprint (PMF) using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS). Multiple sequence alignments were performed by ClustalW, the Bioedit software. Molegro Data Modeller (MDM) 3.0 software was used to predict the putative tertiary structure of the peptide. The enzyme possessed fibrinogenolytic, procoagulant, and aggregation inducer properties. Moreover, the SDS-PAGE (12%) was applied to examine fibrinogenolytic function. The purified enzyme degraded the Aα chain of fibrinogen while the Bβ and γ chains were not digested. According to that, the deficient human plasma in factor X and normal human plasma were also coagulated by TLIECV, it takes part in the common and intrinsic routes of the coagulation cascade. These findings proved that TLIECV is a serine protease identical to procoagulant thrombin-like snake venom proteases; however, it specifically releases the Aα chain of bovine fibrinogen. Because of its function to make up for the deficiency of factor X and its platelet aggregation inducer property, TLIECV could be considered a molecular impact to reveal the hemostasis mechanisms.

Keywords: Blood coagulation, Platelet aggregation inducer, Platelet function, Snake venom, Thrombin-like

1. Introduction

Snake venoms belong to the Viperidae family representing several enzymatic and non-enzymatic proteins responsible for causing hemostatic disturbances. These disturbances are appeared by coagulant disorder, myonecrosis, edema, hemorrhage, local tissue demolition, fibrinogenolysis, thrombosis, and limb amputation (1, 2). One of these agents handles for *in-vitro* procoagulant function, which exists in numerous snake venoms, is serine proteases (SVSPs), which functionally and structurally resemble at least in part to thrombin. This multifunctional protease that has a vital role in the coagulation cascade is called snake venom thrombin-like enzymes (SVTLEs). Thrombin-like enzymes have pro-coagulate, proteolytic, platelet aggregation inducer, and hemorrhagic properties, which interact with the mechanism of coagulation. These enzymes are similar to thrombin (IIa) in their activity to form the clot of fibrinogen via fibrinopeptide digest. Some other SVSPs, although missing the fibrinogen-clotting property, can directly aggregate platelets in washed platelet suspensions. Because of their golden roles in the homeostasis system, such as preventing thrombi formation and improving blood circulation by reducing blood viscosity, SVSPs have been considered drugs in the clinical therapeutic area in cardiovascular diseases and hemostatic disorders, such as congestive heart failure, ischemic stroke, thrombotic disorders, etc. (3, 4). *Ancrod* and *Batroxobin* purified from the *Agkistrodon rhodostoma (Calloselasma rhodostoma)* and *Bothrops atrox moojeni* venoms, respectively, are examples of SVSPs that have been introduced as drugs. The study of thrombin-like enzymes purified from snake venoms creates opportunities for understanding the structural details of their highly specific coagulant functions on fibrinogen (5-8). The present research, for the first time, reports on the purification by a single-step chromatographic protocol, the characterization of some biochemical properties, and the identification of a thrombin-like enzyme from IECV, a special *Viper,* found in Iran, using both its intact molecular mass and peptide mass fingerprint (PMF) by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS).

2. Materials and Methods

2.1. Materials

The bovine serum albumin, Acetonitrile (HPLC grade), Water (HPLC grade), Tris-buffer, Tris-Base, Calcium chloride (CaCl2), Na2CO3, CuSO4:5H2O, NaOH, Folin reagent, Sodium potassium tartrate, Sephadex G-50, Ammonium acetate, Trifluoroacetic acid (TFA), Foline, DEAE-Sepharose, CM-Sepharose, EDTA (Ethylenediaminetetraacetic acid), Heparin, S2238, Ticlopidine, Clopidogrel, Aspirin, other chemicals and reagents used were analytical grade from Merck (Merck Millipore, Darmstadt, Germany). The Prothrombin Time (PT), Activated Partial Thromboplastin Time (APTT), STA®-Thrombin Kit and Fibri-Prest® 2 Kit were purchased from Fisher Diagnostics (USA), and all other chemicals used were of the highest quality available.

2.1.1. Mass Spectrometry

Alpha-cyano-4-*hydroxy*-cinnamic acid (CHCA), Acetonitrile (ACN) (MASS grade) and TFA and other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan).

2.1.2. Biological Material and Venom Extraction

The Lyophilized Crude venom of IEC was obtained from the Department of Venomous Animals and Antivenom Production, Razi Vaccine and Serum Research Institute Karaj, Iran.

2.2. Fractionation of IECV through RP-HPLC Chromatography

A total of 7 mg of IECV (dry weight) was dissolved in 70 mM Tris-Cl, pH-7.4 buffer containing 250 mM NaCl (buffer A) and centrifuged at 10,000 rpm for 15 min. The supernatant was filtered through a 0.2 µ membrane syringe filter, and protein concentration was measured by the NanoDrop® (9). Then, 0.1 ml filtrate containing 3 mg of protein was fractionated on an HPLC system (600, Waters, USA) equipped with a

C_{18} column (5 µm, 9.4 ×250 mm, Agilent Inc., USA). The column was pre-equilibrated with the aforementioned buffer, and the flow rate was adjusted to 0.5 ml/min^{-1} at room temperature (~23°C) over 95 min. Excitation wavelengths of 215 and 280 nm were applied to monitor the different peptide fraction peaks, and fractions of 0.5 ml were pooled. The fractions were concentrated using a Micro-concentrator (Amicon), and were prepared for the pharmacological, enzymatic, and MALDI-TOF/MS assays.

2.3. SDS-PAGE Analysis of IECV

The IEC crude venom was examined using 12.5% SDS-PAGE as non-reduced and reduced conditions.

2.4. Plasma Clotting Time (PCT), Recalcification Time, Prothrombin Time (PT), and Activated Partial Thromboplastin Time (APTT)

The abovementioned assays were performed as described previously (10).

2.5. Thrombin Clotting Time and Fibrinogen-clotting Activity

Thrombin clotting time (TCT) was determined using a commercial kit (*STA®-Thrombin kit (REF 00611)* and Manual coagulation analyzer PKL PPC 170). Thrombin Clotting Time was measured after incubating 100 *µl* plasma at 37°C for 2 min. Thrombin time was recorded by a Manual coagulation analyzer after adding 100 µL of mixed thrombin reagent and sample (Crude venom samples/fractions). Fibrinogen-clotting activity (FCA) was determined using a commercial *Fibri-Prest® 2 Kit (00608,100045),* based on its instructions in the water bath. Briefly, 100 µl fibrinogen reagent and 200 µl of diluted human plasma (1:10 concentration/pH=7.4) were incubated at 37°C for 2 min. Next, 200 µl plasma, 100 µl of crude venom samples/fractions, and 100 µl fibrinogen reagent were mixed and shaken in a water bath. The fibrinogen concentration was estimated from a fibrinogen standard DIAGNOSTICA STAGO *(100045).*

2.6. Fibrinogenolytic Activity

Different amounts of purified fraction (F_{11}) were added to the 60 µl bovine fibrinogen with 1 (mg/ml)concentration dissolved in PBS (Phosphate-buffered saline) (pH 7.2) at 37°C for 1, 6, 12, and 24 h. Fibrinogen without F_{11} fraction treatment was considered as a control. Aliquots of the reaction mixture were then examined by 12% SDS-PAGE (1).

2.7. Effect of Thrombin-like Enzyme on Deficient Human Plasmas

Factor Xa-like activity was measured using factor X deficient plasma. Deficient factor X, II, and V deficient plasma (100 ml) was mixed with the purified peptide (50µL) at 37°C, and the clot formation was recorded.

2.8. Effect of Thrombin-like Enzyme on Platelet Aggregation

Donors should not consume drugs to interfere with platelet reactions, such as Aspirin and other NSAIDs (Nonsteroidal Anti-Inflammatory Drugs) or Thienopyridines (e.g., Ticlopidine and Clopidogrel) two weeks before the experiment. Blood samples were collected from a forearm vein with a vacuum syringe equipped with 8.5 mL plastic centrifuge vacuum tubes containing 1.5 volumes of acid-citrate-dextrose anticoagulant. The tubes are immediately placed in a water bath at 37°C for 15 min. Washed platelet preparation was carried out according to the modified Preparation of Washed Human Platelets (Platelet aggregation using washed human platelets) method. Platelet aggregation was measured in a whole the CHRONO-LOG® Model 700 Whole Blood/Optical Lumi-Aggregometers (Pennsylvania, USA). Assays were performed in siliconized glass cuvettes under stirring. Aggregation was started by adding Collagen, Arachidonic acid, ADP (Adenosine diphosphate), Thrombin agonists (Control), crude venom or purified enzyme (Sample1), mixed Collagen or Arachidonic acid or ADP or Thrombin agonists or Clopidogrel or Ticlopidine or Aspirin drugs and a purified fraction (Sample2) to human washed platelets. The amount of 100% aggregation was recorded with supra-maximal Collagen, ADP, Thrombin, or Arachidonic acid agonists concentrations.

2.9. Effect of Antagonists on Platelet Aggregation Induced by Thrombin-like Enzyme

Drugs, including acetylsalicylic acid (aspirin) (150

mmol/l), irreversible inhibitor of cyclooxygenase-1 (COX-1), ticlopidine (ticlid), and clopidogrel (Plavix) antagonists of P2Y12 receptor (150 mmol/l) were used to assess platelet receptors on the platelet aggregation action.

2.10. Mass Spectrometry and Database Searching

2.10.1. MALDI-TOF/MS Method

The gel was fixed in a solution containing 45% deionized water, 45% methanol, and 10% acetic acid for 2 h. Coomassie blue color has been applied for highlighting separated bands on gel electrophoresis overnight. After this time, single bands of F_{11} were extracted and placed into Eppendorf tubes. In-gel digestion protocol using trypsin was applied to produce peptide segments that have been broken at the lysine-arginine bonds (11, 12). Based on molecular mass data, prediction of the partial structure of F_{11} was obtained using MALDI-TOF/MS analysis. The obtained m/z values of the peptide ions of the F_{11} were matched to the Mascot database to identify some matched peptides. The MALDI-TOF/MS (Applied Biosystems 4800 Nd: YAG 200-HZ laser) was used for MS (Mass spectrometry) experiments. For MS analysis, the digested sample was spotted on a MALDI plate mixed with alpha-cyano-4-*hydroxy*-cinnamic acid CHCA in 50% ACN containing 0.1% TFA (with a 1:2 ratio respectively) as a matrix solution, air dried, and analyzed in positive reflector mode (the mass range was 800-4000 Da). The MALDI-TOF/MS data were interpreted and processed using the Mascot database.

2.11. Alignment

The Clustal W, Bioedit 7.2.6, and BLASTP algorithm (blast.ncbi.nlm.nih.gov/Blast.cgi) methods were used to homology search and analyze the multiple sequence alignment.

2.12. Bioinformatics Analysis

Predicted putative three-dimensional structures of the F_{11} fraction according to the data obtained from Mascot analysis were modeled by Molegro Data Modeller (MDM) 3.0 software.

2.13. Statistical Analysis

The general linear model procedure in SAS 9.1 software was applied to examine the significance of the difference in Activated Clotting Time and pharmacological assays of IECV and its fractions as control. The values are reported as the Mean ± SD. A $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Purification of the Thrombin-like Enzyme from Iranian *Echis carinatus* Venom

The purification was modified by a single affinity chromatography step, and the results are shown in Fig 1a. The fractionation of IECV was performed by one chromatographic step, resulting in the isolation of the procoagulant and aggregation inducer fraction. The profile of the single chromatographic step on the C_{18} reverse-phase column showed eleven fractions (Fig. 1a). Eleven fractions were obtained named F_1 to F_{11}. In order to confirm the pure peak, additional extra purification was performed by RP-HPLC on a C_{18} column which led to a significant corresponding purified fraction (Fig.1b).

3.2. Coagulant Activity of TLIECV on Human Plasma

Coagulant activity evaluation of all fractions have been recorded in Fig 2 a, b, c, d, e, and f. The F_{11} fraction was found to be a potent procoagulant fraction and was used for more analysis. A significant coagulation of human plasma induced by the F_{11} fraction was observed, as well as for the whole venom. Although the coagulant function assay revealed that all doses of the F_{11} fraction samples had procoagulant properties, they showed different coagulation times (Fig. 3).

3.3. SDS-PAGE Analysis of IECV

The SDS-PAGE analyses of crude venom under reduced and non-reduced conditions were shown in Fig.4a. The analysis of the protein bands from IEC crude venom suggested the majority of proteins in the mass ranges of 55–90 kDa and 10–40 kDa. The purified enzyme with procoagulant function (F_{11}) was visualized as a single band in gel-electrophoresis with a molecular mass of 3 kDa (Fig. 4b).

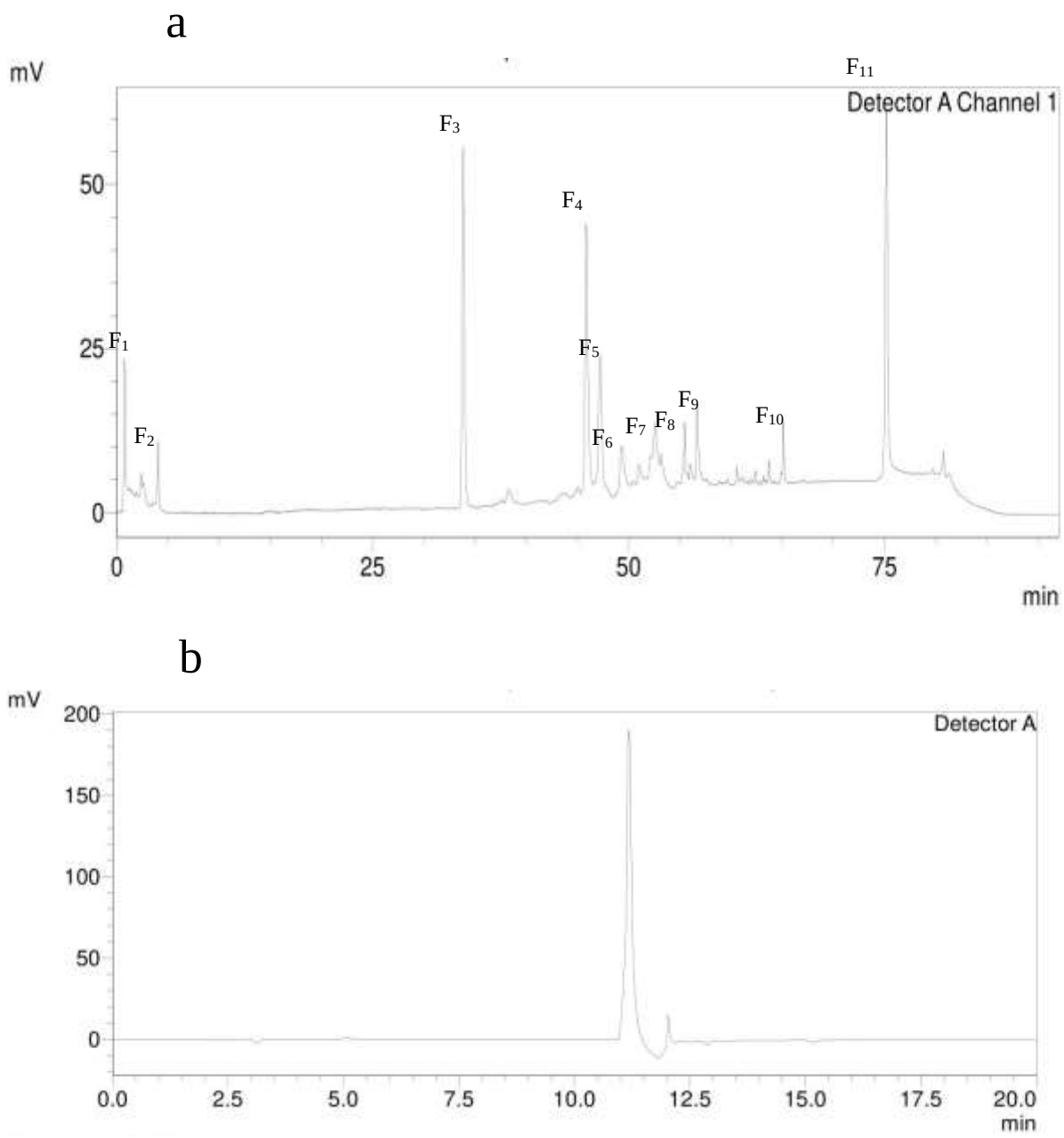


Figure 1. a: RP-HPLC chromatography profile of the soluble venom of IEC. The column elution profile exhibited eleven peaks (F_1-F_{11}). Fractionation of crude venom by reverse-phase-HPLC using column C_{18} equilibrated with trifluoroacetic acid (0.1%) (Solvent A). The eluted material was monitored at 280 nm. b: Confirmation of the pure peak by RP-HPLC on a C_{18} column

3.4. Fibrinogenolytic Activity on Purified Bovine Fibrinogen

The findings of fibrinogenolytic test showed that the TLIECV selectively degraded fibrinopeptide A of fibrinogen while fibrinopeptide B and γ chain were unchanged even after 24 h (Fig. 4c).

3.5. Effect of Thrombin-like Enzyme on Deficient Human Plasmas

The coagulant activity of TLIECV was also assayed on factor II, X, or V deficient human plasmas; TLIEC

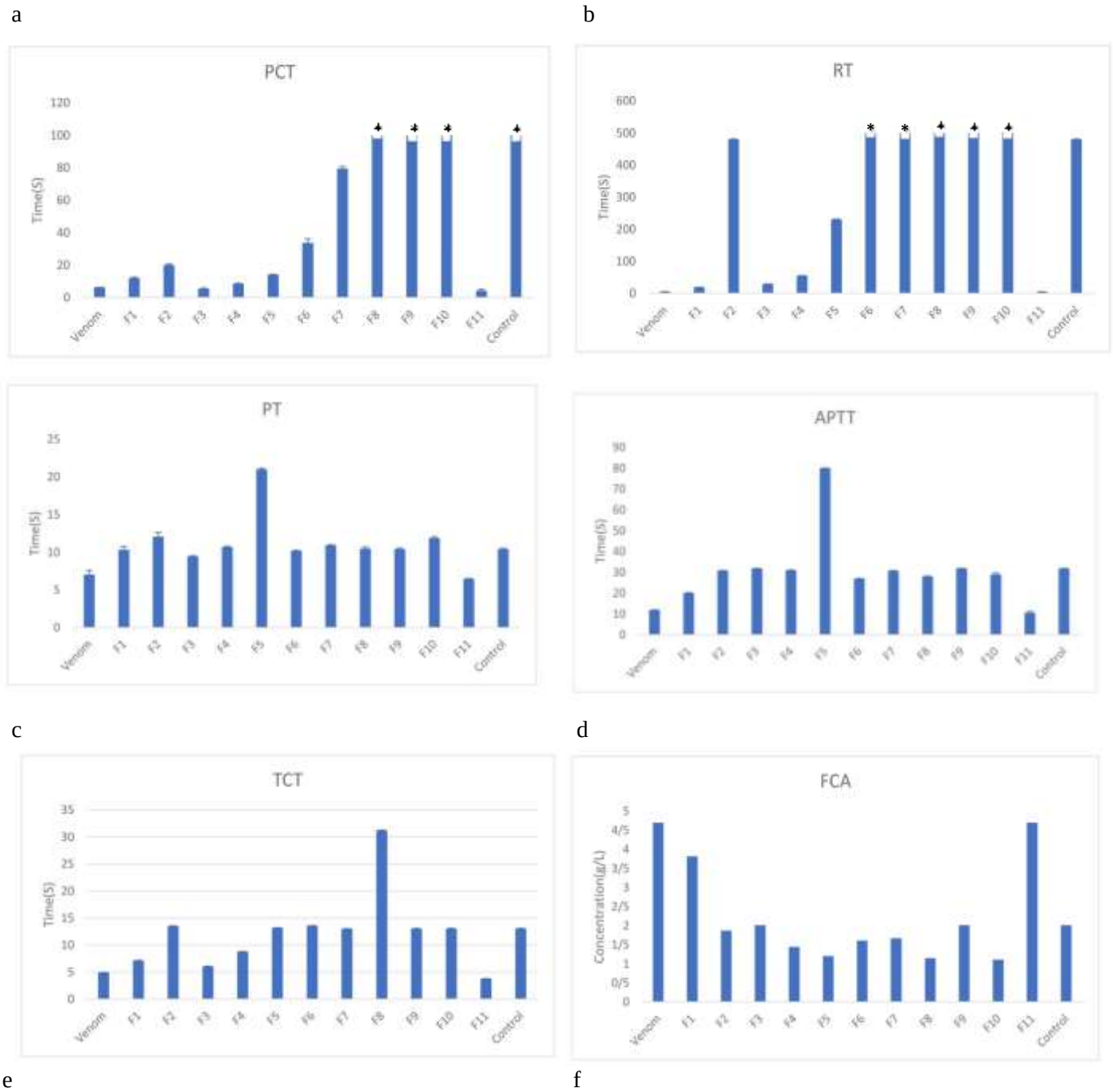


Figure 2. Effect of TLIECV on clotting plasma. Data expressed as mean ± SD.a: PCT. b: RT. c: PT. d: APTT. e: TCT. f: FCA. *: No Clot

seems to prolong the CTs for deficient plasmas in II and V factors. The purified enzyme decreased the coagulation time of factor X-deficient human plasma (Table 1). Increased deficient plasmas in factors II and V plasma coagulation time proposed that TLIECV could not compensate for the lack of these agents.

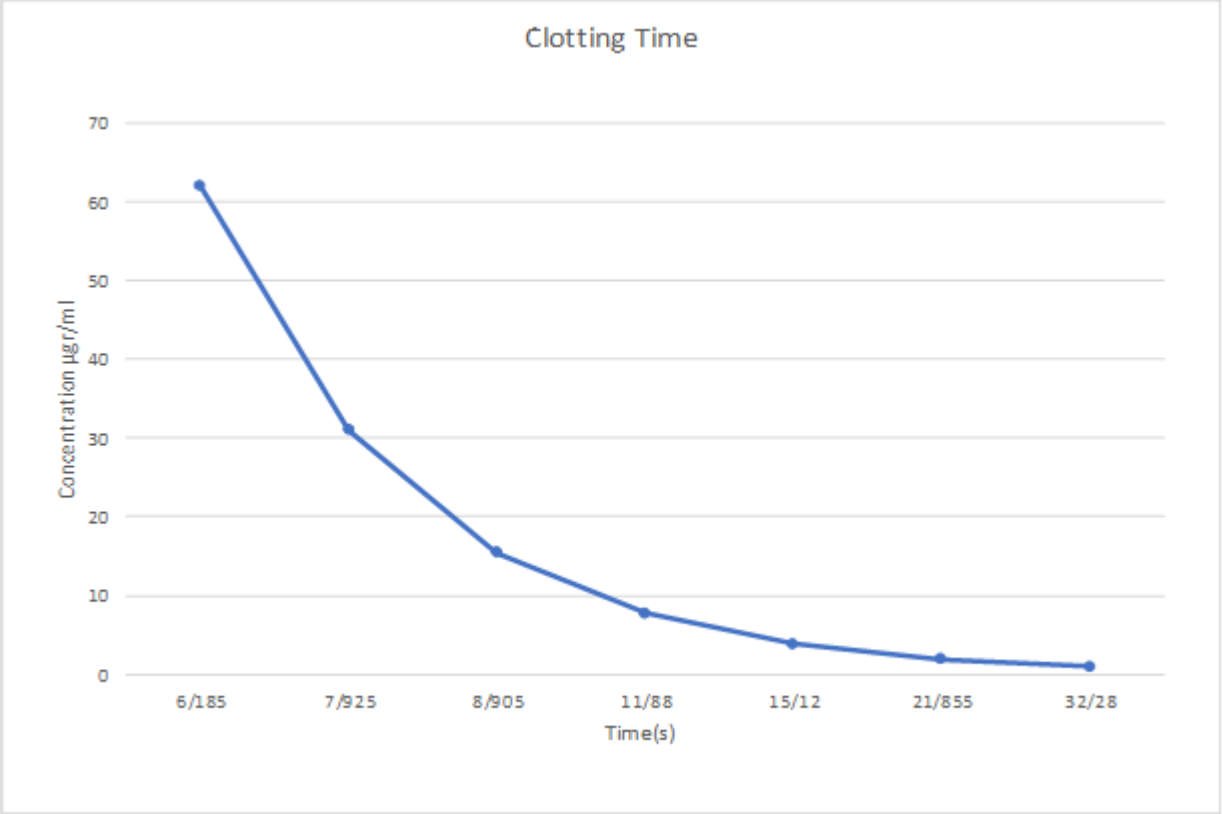


Figure 3. Coagulant activity of TLIECV

3.6. Effect of Thrombin-like Enzyme on Platelet Aggregation

The effect of TLIECV on platelet aggregation was examined on human-washed platelets. The obtained data revealed that this peptide induced a maximal platelet aggregation (86%), such as ADP, arachidonic acid, collagen, and thrombin agonists (Fig. 5 a, b).

3.7. Effect of Antagonists on Platelet Aggregation Induced by Thrombin-like Enzyme

Using Ticlopidine (86%) reduced the platelet aggregation function of TLIECV compared with the Clopidogrel effect with 80% reduced activity. Aspirin (acetylsalicylic acid) did not affect the platelet aggregation function of TLIECV. These results clarify that Ticlopidine had a more obvious inhibitory effect on the platelet aggregation action of TLIECV than Clopidogrel, which confirmed the involvement of the ADP signaling pathway to initiate platelet aggregation by TLIECV (Fig. 5c).

3.8. MALDI-TOF/MS and Bioinformatics Analysis

Identification of the procoagulant and platelet aggregation inducer fraction purified from IECV was revealed by the MALDI-TOF/MS data analysis in the Mascot format database. The outcomes from MALDI-TOF/MS was displayed in Fig.6a. The PMF analysis was performed for the F_{11} fraction. The results of PMF have confirmed 1 peptide, which is matched to the F_{11} fraction (Fig.6b).

3.9. Alignment

The highly considerable homology of the purified peptide with *Viperidae* venom protein families in protein databases was shown by multiple sequence alignment (Fig. 6c). The sequence of TLIECV was compared with those of other serine proteases from snake venoms (Table 2).

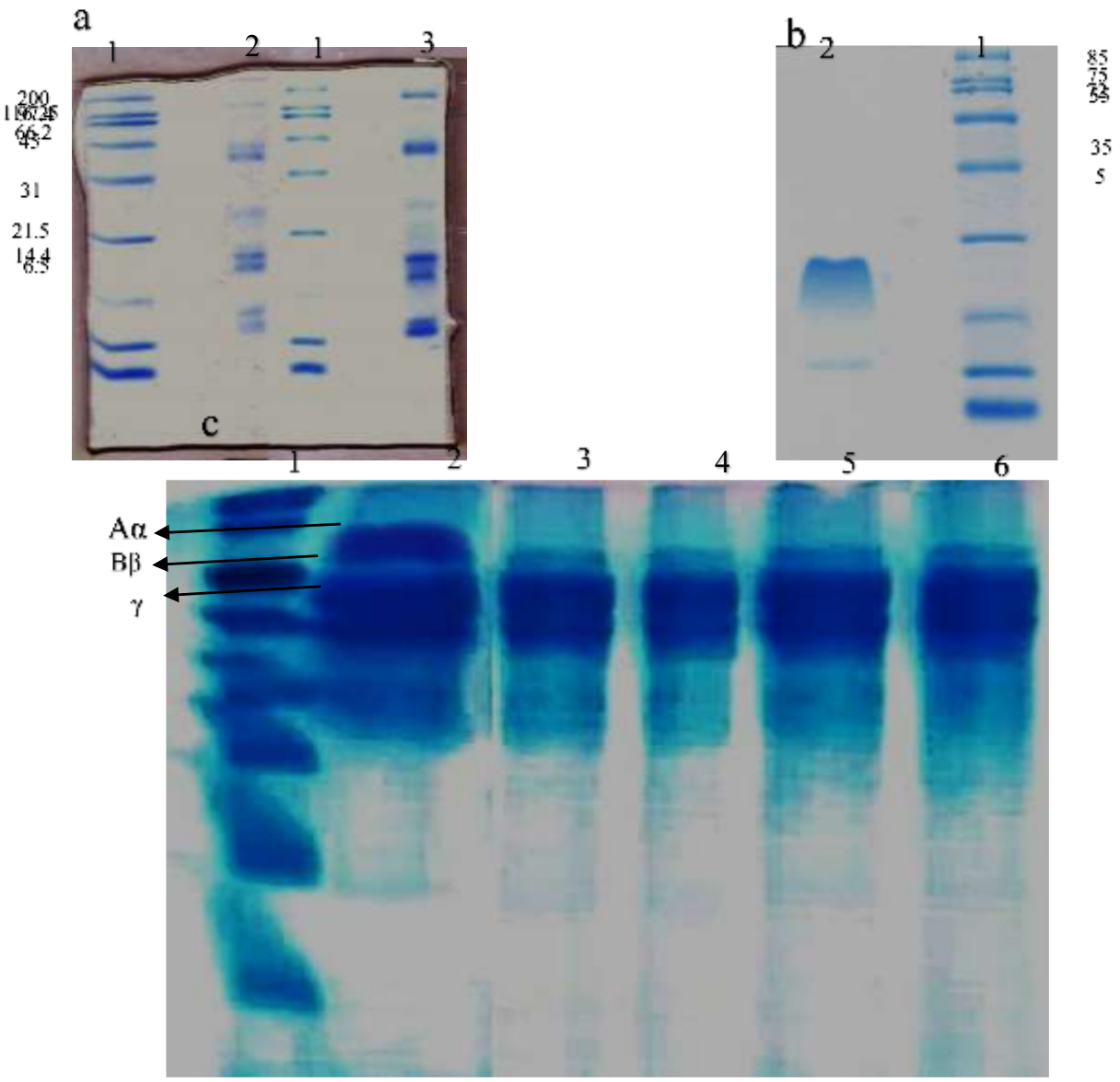


Figure 4. a: SDS-PAGE analysis of IEC crude venom. Lane 1, 2 and 3 represent protein markers, IEC crude venom (reduced), and IEC crude venom (non-reduced) respectively. b: Determination of the molecular mass of TLIEC by SDS–PAGE analysis (12.5%). Lane 1, molecular mass markers, Lane 2. TLIEC. c: TLIEC incubated with fibrinogen (3 mg/ml) at different times (1, 6, 12 and 24 h), the activity was evaluated using SDS–PAGE (12%), Lane 1, Molecular mass markers Lane 2, Fibrinogen control, Lane 3–6, different times (1, 6, 12 and 24 h), respectively.

Table 1. Effect of TLIECV on the clotting time of deficient human plasmas

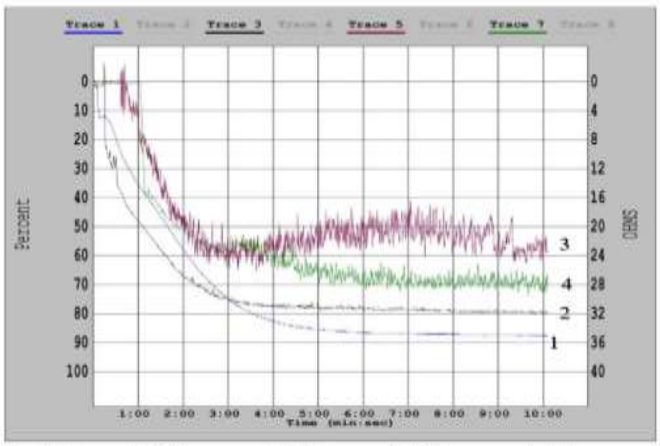
Plasma	Time of reference (s)	Time with TLIECV(s)
Normal	13	6.21
Deficient in factor II	79	376
Deficient in factor V	80	167
Deficient in factor X	336	68

The plasma was mixed with purified enzyme and incubated at 37°C. Data are expressed as mean ± SD (n = 3)

Table 1. Effect of TLIECV on the clotting time of deficient human plasmas

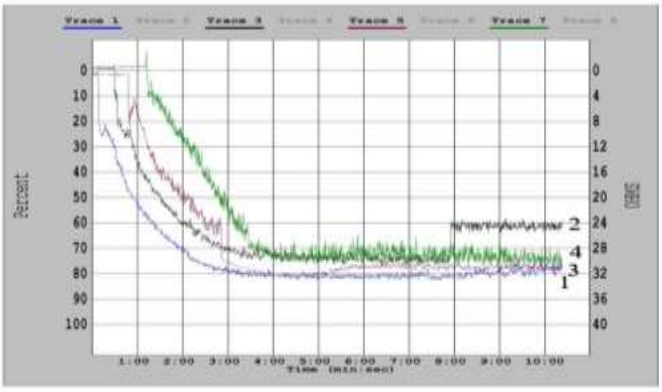
Plasma	Time of reference (s)	Time with TLIEC(s)	*P* value
Normal	13.19±0.3	6.13±0.11	< .00001
Deficient in factor II	79.23±0.29	375.08±1.66	< .00001
Deficient in factor V	80.23±0.22	166.67±0.58	< .00001
Deficient in factor X	335.61±0.53	67.72±1.44	< .00001

The plasma was mixed with purified enzyme and incubated at 37°C. Data are expressed as mean ± SD (n=3)



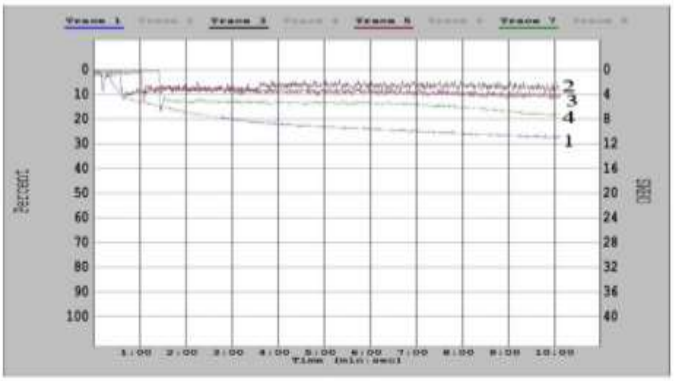
	1	2	3	4
Amplitude	86%	78%	65%	73%
Slope	46	125	75	149
Lag Time	0:05	0:06	0:23	0:09
Area Under	378.6	391.5	274.7	345.6

a



	1	2	3	4
Amplitude	82%	77%	80%	78%
Slope	99	105	72	64
Lag Time	0:06	0:06	0:06	0:10
Area Under	408.4	378.6	381.9	351.1

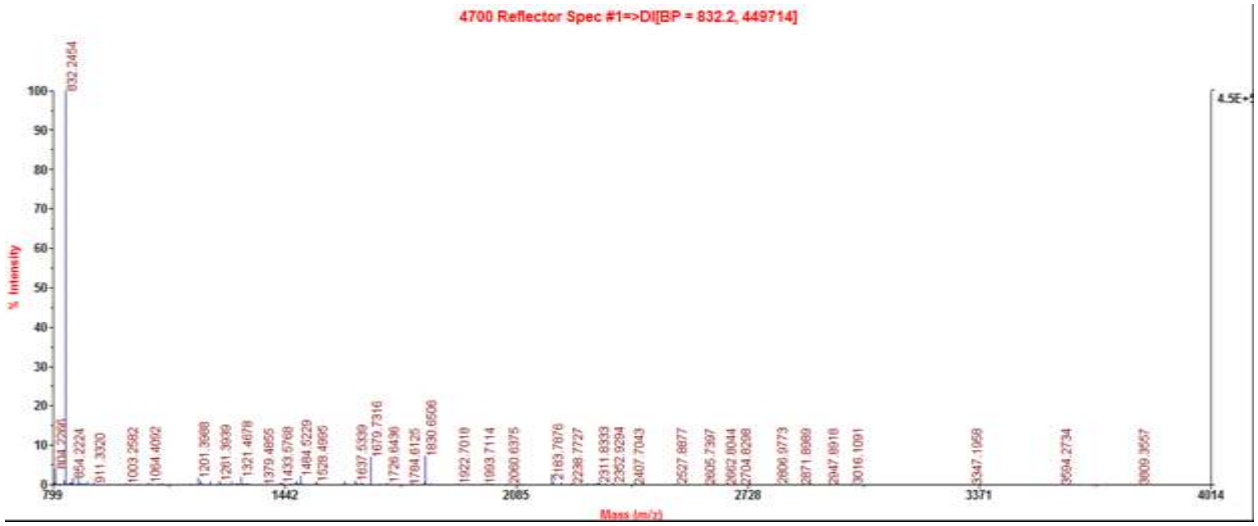
b



	1	2	3	4
Amplitude	23%	10%	12%	17%
Slope	29	31	28	48
Lag Time	0:14	0:04	0:11	0:09
Area Under	102.3	31.3	41.3	75.3

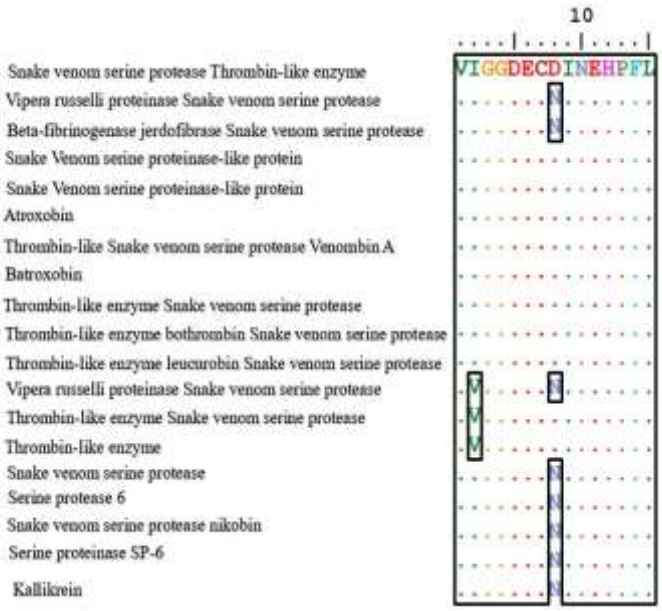
c

Figure 5. Effects of the purified fraction on the platelet aggregation in washed human platelets suspension. a: 1. Washed platelets + Arachidonic Acid+ Normal saline 2. Washed platelets + Collagen + Normal saline 3. Washed platelets + ADP + Normal saline 4. Washed platelets + Thrombin + Normal saline). b: 1. Washed platelets + (TLIECV) + Normal saline 2. Washed platelets + (TLIECV+ AA) 2. Washed platelets + (TLIECV + Collagen) 2. Washed platelets + (TLIECV+ Aspirin). c: 1. Washed platelets + (TLIECV + Clopidogrel) 2. Washed platelets + (TLIECV+ Ticlopidine)



a

b



c

e

Figure 6. a: Mass spectrum profile of TLIECV. (b) PMF analysis for identification of TLIECV, (c): Alignment of the predicted amino acid sequences of TLIECV peptide with Viperidae venom protein families in protein databases. This alignment is based on the comparison with the sequence; this sequence has the most similarity with the sequence obtained from the Mascot analysis of the MASS results of the putative peptide using Bioinformatics analysis. Sequence alignment between purified peptide sequences and the closest BLAST match. (d): Predicted three-dimensional structure of TLIECV resulting from bioinformatics analysis

Table 2. Amino acid sequences of TLIECV and other serine proteases.

TLIECV	V I G G D E C D I N E H P F L
Purpurase	V V G G D E C N I N E H R S L V A I F (20)
Catroxobin	V V G G D E C N I N E H R S L V A I F (23)
Flavoxobin	V I G G D E C D I N E H P F L V A L (21)
Crotalase	V I G G D E C N I N E H P F L – A F (24)
Gabonase	V V G G A E C K I D G H R C L V A L Y (25)
Thrombin	I V E G Q D A E V G L S P W Q V M L F (26)
Kallikrein	V V G G Y N C E M N S Q P W Q V A V Y (27)
Trypsin I	V G G Y T C G A N T V P Y Q V S L N (28)

3.10. Bioinformatics Analysis

The nearest 3D structure of TLIECV resulting from bioinformatics analysis according to the data obtained from Mascot analysis is shown in Fig.6d.

4. Discussion

Preservatives have been employed in the seafood industry to prolong the shelf-life of commercial products. Microalgae are a valuable source of extracts and bio-preservative compounds that may not be available in other raw materials. Microalgae are a rich source of major bioactive compounds such as proteins, fatty acids (primarily omega-3), pigments, carotenoids, polysaccharides, and vitamins. These compounds have significant value in seafood production, as they can improve the quality and extend the shelf-life of the product. According to a study (22), extracts of microalgae possess bioactive compounds that have the potential to compromise the structural integrity of bacterial cell membranes. The active compounds have the potential to enhance the permeability of cell membranes, leading to the significant loss of essential ions, including potassium and other cytoplasmic components. This phenomenon has the potential to ultimately result in cellular demise.

Phycobiliproteins are hydrophilic protein-pigment complexes that exhibit hydrophilic properties. They are found in various cyanobacterial species, including *Phorphyridium*, *Spirulina*, and *Oscillatoria*. The mentioned pigments, which are primarily composed of proteins, have been categorized into three distinct groups: phycocyanins (blue pigments), allophycocyanins (pale-blue pigments), and phycoerythrins (red pigments). The utilization of these pigments as natural colorants and bioactive agents in seafood processing has been documented (23, 24).

The utilization of microalgae biomass in seafood has been observed to improve quality and prolong shelf-life by mitigating microbial growth and chemical reactions. In a recent investigation conducted by Ben Atitallah et al. (2019) (25), it was demonstrated that the inclusion of *Chlorella minutissima*, *Isochrysis galbana*, and *Picochlorum* sp. powder in canned fish burgers made from common barbel (Barbus barbus) resulted in a significant enhancement of the overall sensory acceptability, texture analysis parameters (namely, hardness, chewiness, gumminess, and cohesiveness), nutritional value, and functional characteristics (specifically, water and oil holding capacities) in comparison to the control group. The results of the microbial contamination analysis indicate the absence of foodborne pathogens, mold, or yeast growth over two months at a temperature of 4˚C.

Compared to our study, total viable microorganisms such as mesophilic, psychrotrophic, and *Staphylococcus aureus* coagulase positive, coliform, and salmonella bacteria were analyzed. In all of these performed tests, the highest total number of bacteria belonged to sample Nile Tilapia fillet

coated with PE solution at a temperature of 8°C and the lowest amount of bacteria (Nile Tilapia fillet) coated with PE solution at a temperature of 4˚C was observed (P≤0.05).

In addition to the chemical and microbial attributes, the sensory characteristics of seafood play a significant role in shaping consumers' preferences. The sensory quality of seafood is significantly influenced by the appropriate color, which is considered an integral attribute. The perception of a pale coloration is frequently indicative of inferior quality, whereas the presence of natural and vibrant hues is subconsciously linked to premium seafood (23). To attain the desired aesthetic qualities and coloration preferred by consumers, it is imperative to optimize the dietary levels of pigments for aquaculture species (23).

Moreover, sensory evaluations, including flavor, texture, color, and total acceptance, were performed. According to the results of the mean comparison, on day zero, no significant statistical difference was observed in the taste score in the samples with and without PE at 4°C and 8°C (P>0.05). Totally, for all of these sensory analyses, the highest score belonged to sample Nile Tilapia fillet coated with PE solution at a temperature of 8°C and the lowest score for Nile Tilapia fillet coated with PE solution at a temperature of 4˚C was observed (P≤0.05).

Homeostasis system is one of the vital systems that is targeted by proteolytic enzymes from snake venoms. In Iran, 90% of snakebite accidents are caused by the viper *Echis carinatus*. The present study explained the purification of a new thrombin-like enzyme purified from IECV with a molecular weight of about 3KDa. This enzyme acts on protein substrates, such as fibrinogen. Although the TLIECV seems to degrade the α-chain of fibrinogen (A-α fibrinogenase), the B-β and γ chains were not digested, indicating that TLIECV is an Aα-fibrinogenase similar to *RP34* and *BbrzSP-32* serine proteinases purified from *C. cerastes* and *B. brazili* venoms, respectively (13, 14). *BjussuSP-I*, *BJ-48*, and *Collinein-1* are examples of SVTLE-A that break down only Aα chain of fibrinogen. Consequently, the TLIECV is a member of SVTLE-A. The SVTLEs are similar to thrombin; therefore, digesting fibrinogen results in to release of the A or B fibrinopeptides. When fibrinogen is digested, two fragments are obtained. Whereas, thrombin breaks down fibrinogen between Arg-Lys link of both the Aα and Bβ chains (15-17). Other thrombin-likes that release fibrinopeptide A and not fibrinopeptide B are batroxobin from *B. atrox*, flavoxobin of *Trimeresurus flavoviridis*, ancrod from *A. rhodostoma*, elegaxobin, and elegaxobin II from *Trimeresurus elegans* venom. Ancrod and batroxobin are currently used as defibrinogenating drugs in patients with vascular occlusive diseases (5-7, 9, 18-21). Because of its specificity to replace the lack of factor X, it confirmed that TLIECV had *in-vitro* procoagulant activity upon citrated human plasma through the beginning of the intrinsic and common routes. The TLIECV displayed fibrinogen clotting activity. The results revealed that TLIECV induced platelet aggregation (86%), such as thrombin. The aggregating ability of TLIECV was highly affected by Ticlopidine compared with Clopidogrel (*P2Y12 ADP* receptor inhibitors), whereas Aspirin (*COX-1* inhibitor) showed no effect (1). These results showed the involvement of the *ADP/P2Y12/Gi* pathway in the aggregating activity of TLIECV. As shown in a sequence alignment, it was closely related to previously known thrombin-like enzymes, TLIECV was homologous in sequence to *atroxobin*, *batroxobin*, and *kallikrein*. Six amino acid residues (e.g., Val, Ile, Gly, Gly, Asp, Glu) and one amino acid (e.g., Leu) were identified as N and C-terminal sequences of TLIECV, respectively. The N-terminal sequence (Val-Ile-Gly-Gly) of TLIECV was the same as the N-terminal sequence of other serine proteinases, such as *elegaxobin* and *elegaxobin II*. Previous studies suggested that the His amino acid residue of TLIECV plays a key role in the clotting activity of this enzyme. Additionally, the Glu residue is an essential amino acid residue since it binds to the target protein (9, 19, 22).

Identification of purified enzyme from IECV confirms that it is a thrombin-like enzyme, exhibiting a procoagulant activity probably through the activation of common and intrinsic pathways. This TLIECV also possesses platelet aggregation inducer effects via *ADP/P2Y12/Gi* signaling. All present data referred to the consideration that TLIECV could be considered as a biological agent to clarify the details of the hemostatic mechanism and design drugs.

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Authors' Contribution

All the authors contributed significantly to this research. N. NN wrote the original draft, prepared figures and tables and was involved in all tests. NM, MA, HV are supervisors. MAB, assisted in the experimental research and in the preparation of the paper. MN assisted in writing the paper and Statistical analysis. All authors have read and approved the final manuscript.

Ethics

Not Applicable.

Conflict of Interest

The authors declare that they have no conflicts of interest to disclose.

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