

DIRECTED PROTEOLYSIS OF FIBRINOGEN BY PROTEASE OF *Gloydius halys halys* VENOM

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One of the approaches for studying structure and functions of proteins is their limited proteolysis. Proteolytic fragments of macromolecules can preserve the biological activity and can be used for the study of their structural and functional peculiarities. Thus, the characterization of new proteolytic enzymes and determination of the specificity of their action can be of interest for exploration. In the present work, we focused on the action of protease from the venom of *Gloydius halys halys* on fibrinogen, the crucial protein of blood coagulation system.

Methods. Products of fibrinogen hydrolysis by protease from the venom of *G. halys halys* were studied by SDS-PAGE electrophoresis and western-blot analysis using monoclonal antibodies II-5 and 1-5A targeted to 20–78 and 549–610 fragments of fibrinogen A α -chain. Molecular weights of hydrolytic products were determined using MALDI-TOF analysis on Voyager DE PRO (USA). Sequence of hydrolytic products were predicted by “Peptide Mass Calculator” software.

Results. SDS-PAGE showed that protease from the venom of *Gloydius halys halys* initially cleaved A α -chain of fibrinogen molecule. Western-blot analysis confirmed that this protease specifically cleaves off fragment of C-terminal parts of A α -chain with apparent molecular weight of 22 kDa. Cleaved fragment was identified by MALDI-TOF analysis as the 21.1 kDa polypeptide. “Peptide Mass Calculator” predicted that such a fragment corresponded to A α 414-610 residue of fibrinogen molecule. Thus, we showed that studied protease cleaved peptide bond A α K413-L414 with the formation of stable partly hydrolyzed fibrinogen desA α 414-610.

Conclusions. The use of protease from the venom of *Gloydius halys halys* would allow obtaining the unique partly hydrolyzed fibrinogen des A α 414–610 that is suitable for the study of structure and functions of fibrinogen α C-regions.

Key words: fibrinogen, limited proteolysis, protease, fibrin polymerization, hemostasis.

Proteinases that exhibit fibrinogenolytic activity can be used to study the structure and function of fibrin(ogen). Limited proteolysis allows to obtain unique fragments of fibrinogen, studying which we can ascertain the functional importance of cleaved sites in the processes of fibrin polymerization, platelet aggregation, endothelial cell proliferation, leukocyte interaction, etc. [1]. Fragments of fibrinogen obtained by limited proteolysis can retain biological activity, information about which is important in medicine primarily for understanding the course of pathophysiological processes [2]. In addition, proteinases targeting

fibrinogen and/or fibrin are also of interest as potential agents for direct defibrination *in vivo* [3, 4]. One of the sources of fibrinogenolytic proteinases is snake venom, which mostly contains α - or β -fibrinogenases [5, 6].

The vast majority of α -fibrinogenases isolated from snake venoms are metalloproteinases with an average molecular weight of 20–26 kDa. During longer incubation, they are also able to break down the β -chain of fibrinogen [7]. Such proteinases have been isolated from venoms of snakes of the genera *Gloydius*, *Daboia* [8], *Deinagkistrodon* [9], *Crotalus* [10], *Trimeresurus* [11], *Agkistrodon* [12], and *Naja* [13].

Most of β -fibrinogenases are serine proteinases. They are able to break down the A α chain of the fibrinogen molecule at a lower rate [6]. Such proteinases have been found in snakes of many genera, such as *Trimeresurus* [14], *Bothrops*, *Lachesis* [15], *Gloydius* [16] and *Hydrophis* [17].

Serine proteinases also include a special group of so-called thrombin-like enzymes. These enzymes hydrolyze fibrinogen, and their action is directed at those bonds that are affected by thrombin, which leads to the formation of fibrin [18]. Such enzymes are known in snakes of the genera *Aghkistrodon*, *Bitis*, *Trimeresurus*, *Cerastes*, *Bothrops*, and *Lachesis* [18, 19, 20].

Previously, a fibrinogen-specific serine proteinase with molecular weight of 28 kDa was obtained from the venom of *Gloydius halys halys* [21]. The aim of our study was to study the specificity of its action on fibrinogen, to identify the generated partially hydrolyzed form of fibrinogen and to determine the hydrolyzed bond.

Materials and Methods

Materials

Proteinase with fibrinogenolytic activity was collected from *G. halys halys* by a previously developed method [21]. Fibrinogen was purified from human blood plasma obtained by adding sodium citrate according to the method described by T. V. Varetskaya [22]. Marker proteins (Thermo Fisher Scientific, USA) and anti-mouse goat antibodies labeled with horse reddish peroxidase (Sigma, USA) were used. Mouse monoclonal antibodies II-5C (anti-A α 20-78) and 1-5A (anti-A α 537-595) were developed and purified in the Department of Protein Structure and Function of the Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine [23, 24].

Directed proteolysis of fibrinogen

Hydrolysis of fibrinogen was performed in the ratio of enzyme:substrate = 1:300 in 0.05 M Tris(2-amino-2-hydroxymethyl-propane-1,3-diol)-HCl buffer, pH 7.4, containing 0.13 M NaCl, at a temperature of 37 °C. The hydrolysis reaction was stopped by adding electrophoresis sample buffer containing 2% SDS, 5% glycerol and 2% β -mercaptoethanol and then boiling the resulting mixture.

SDS-PAGE/ Western blot

Electrophoresis was performed by the Laemmli method [25] using the Tris-glycine system. Separation of proteins was performed at a current of 19 mA in the concentrating and

35 mA for the separating gels. Samples for electrophoresis were prepared by adding the protein solution to buffer sample, which was prepared by adding 5% sucrose, 2% SDS and 0,2% bromophenol to the electrode buffer. Before applying to the gel, the samples were heated to boiling.

After SDS-PAGE, the proteins were transferred to the nitrocellulose membrane by electric current (voltage 100 V) for 2 hours. The membrane was blocked with 5% skim milk solution in TBS (tris-buffered saline) for 1 hour. Then the proteins were incubated with mouse monoclonal antibodies II-5C (anti-A α 20-78) or 1-5A (anti-A α 537-595) for 2 hours, and after washing in TBS, incubated with the secondary secondary anti-mouse goat antibodies labeled with peroxidase. The strips were incubated in a solution of 0.001 M 4-chloro-1-naphthol in 0.5 M Tris pH 7.5 and 0.03% H₂O₂ to develop specific immunostaining.

Obtaining low molecular weight products of hydrolysis

To obtain a low molecular weight product of hydrolysis, a solution of fibrinogen (15 mg/ml) in Tris-HCl buffer pH 7.4 with 0.13 M NaCl was incubated for 30 min with proteinase from the venom of *Gloydius halys halys* (0.02 mg/ml) at temperature 37 °C. The hydrolysis reaction was stopped by salting out fibrinogen in an equal volume of 16% Na₂SO₄. After centrifugation at 3 000 rpm for 30 min, the supernatant containing the enzyme and the cleaved polypeptide was collected and examined using mass spectrometry analysis.

Mass spectrometry

Mass spectrometry analysis was performed on a MALDI-TOF spectrometer Voyager DE PRO (Applied Biosystems, USA). H⁺-matrix ionization was performed by laser irradiation. The concentration of sinapine acid (Sigma, USA) in the matrix reagent was 1 mg/ml. The reagent was dissolved in a solution containing equal volumes of acetonitrile (Sigma, USA) and 1% aqueous trifluoroacetic acid (Sigma, USA). The obtained spectra were processed by Data Explorer 4.0.0.0 (Applied Biosystems) [26].

Statistical processing and bioinformatics analysis

Statistical processing of the results was performed using standard statistical software "Microsoft Excel" for Windows 2000.

Determination of the amino acid sequence, cleaved from fibrinogen by the action of proteinase from the venom of *Gloydius halys halys*, was performed with the program "Peptide Mass Calculator" (<https://www.peptidesynthetics.co.uk/tools/>).

Results and Discussion

Fibrinogen-specific proteinase from the venom of *G. halys halys* was obtained by a method previously developed at the Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine. It was found that the studied enzyme has a molecular weight of approximately 28 kDa and belongs to serine proteases, because the activity of the enzyme is inhibited by DFP (diisopropylfluorophosphates) and benzamidine [21].

SDS-PAGE showed that the enzyme from the venom obtained from *G. halys halys* is specific to the A α chain of fibrinogen, which was hydrolyzed within 90 min. No cleavage of B β - and γ -chains was observed during the incubation period. The reaction revealed the accumulation of hydrolysis products of the A α -chain of fibrinogen, the molecular weights of which are approximately 44, 20 and 17 kDa (Fig. 1).

Western blot analysis was performed to determine which part of the A α -chain of fibrinogen is cleaved by proteinase obtained from *G. halys halys*. Accumulation of a high molecular weight polypeptide with a mass of approximately 43 kDa was shown using II-5C monoclonal antibody to the N-terminal portion of the A α chain of the fibrinogen molecule (Fig. 2). When using antibody 1-5A to the C-terminus of the A α -chain of fibrinogen, the formation of two products was observed, with molecular weights of approximately 22 and 18 kDa (Fig. 3).

The obtained results indicate that this

proteinase cleaves a fragment with a molecular weight of approximately 22 kDa from the C-terminus of the A α -chain of fibrinogen.

Detection of two products by Western blot analysis using antibody 1-5A to the C-terminal region of the A α -chain of fibrinogen is due to partial hydrolysis of fibrinogen with low amounts of plasmin impurities. In this case, plasmin cleaves the sequence which includes 27 amino acid residues. As a result, the solution contains a small amount of partially hydrolyzed fibrinogen, the molecular weight of which is 2.8 kDa less than the weight of native fibrinogen [27]. Therefore, two polypeptides can be detected among the hydrolysis products under the action of proteinases that are specific for one peptide bond of the C-terminal region of the A α -chain.

To determine the site of enzymatic hydrolysis of fibrinogen, the exact mass of the obtained polypeptides was determined using MALDI-TOF analysis (Fig. 4). It was found that the hydrolysis produces peaks in which the ratio of mass to charge (M/Z) is 21109 and 18375, which indicates the appearance of polypeptides with masses of 21.1 kDa and 18.4 kDa.

According to calculations in the Peptide Mass Calculator program, this molecular weight corresponds to the peptides A α 414-610 and A α 414-583, which are formed by hydrolysis of the A α Lys413-Leu414 bond of the fibrinogen molecule.

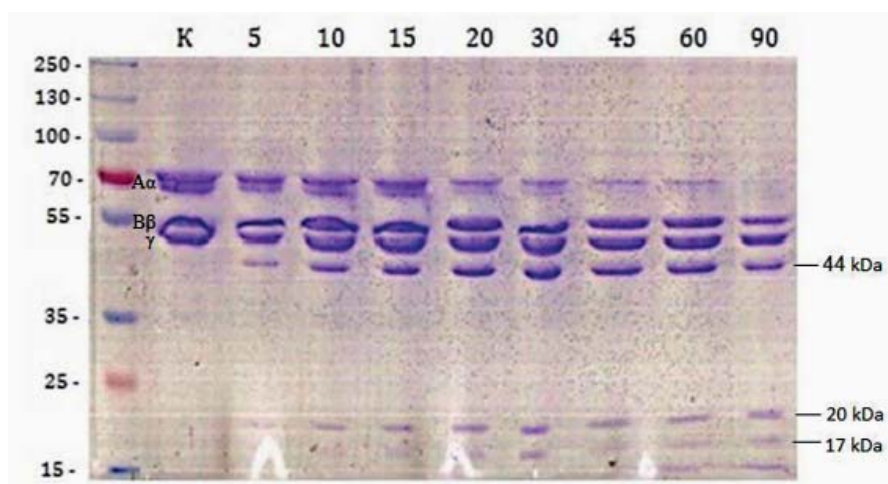


Fig. 1. Electrophoregram of fibrinogen hydrolysis products by proteinase from the venom of *G. halys halys*:

K — native fibrinogen; 5, 10, 15, 20, 30, 45, 60, 90 minutes of exposure to the enzyme. The samples were prepared in the presence of 0.2% β -mercaptoethanol. The gel was stained with Coomassie blue

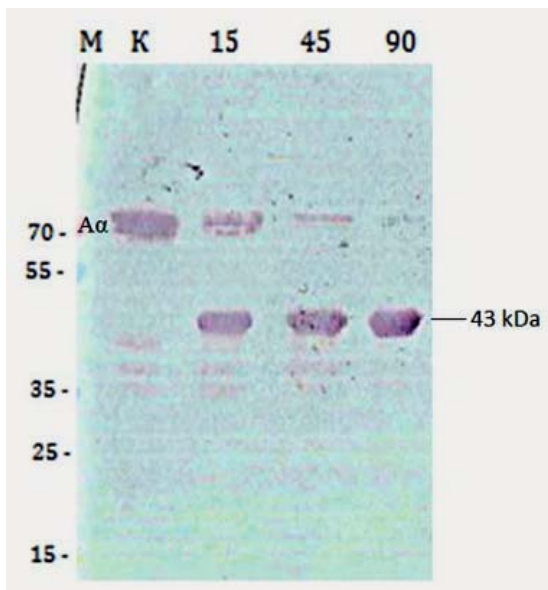


Fig. 2. Western blot analysis of products of fibrinogen hydrolysis by proteinase from the venom of *G. halys halys* using monoclonal antibody II-5C to the N-terminal regions of the A α -chain of fibrinogen:

K — native fibrinogen; 15, 45, 90 minutes of exposure to the enzyme. Samples were prepared in the presence of 0.2% β -mercaptoethanol

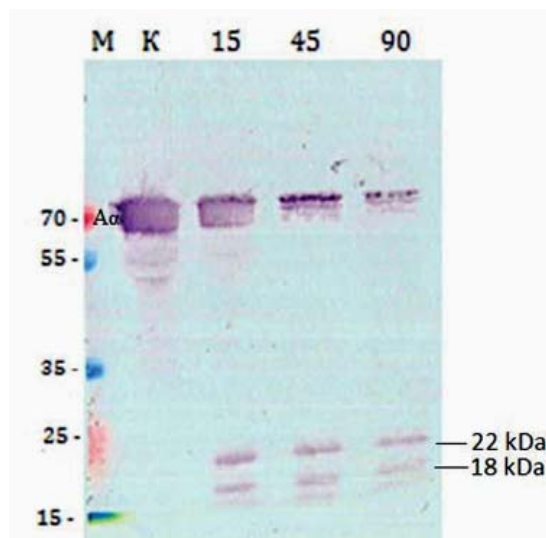


Fig. 3. Western blot analysis of products of fibrinogen hydrolysis by proteinase from the venom of *G. halys halys* using monoclonal antibody I-5A to the C-terminal regions of the A α -chain of fibrinogen:

K — native fibrinogen; 15, 45, 90 min of exposure to the enzyme. Samples were prepared in the presence of 0.2% β -mercaptoethanol

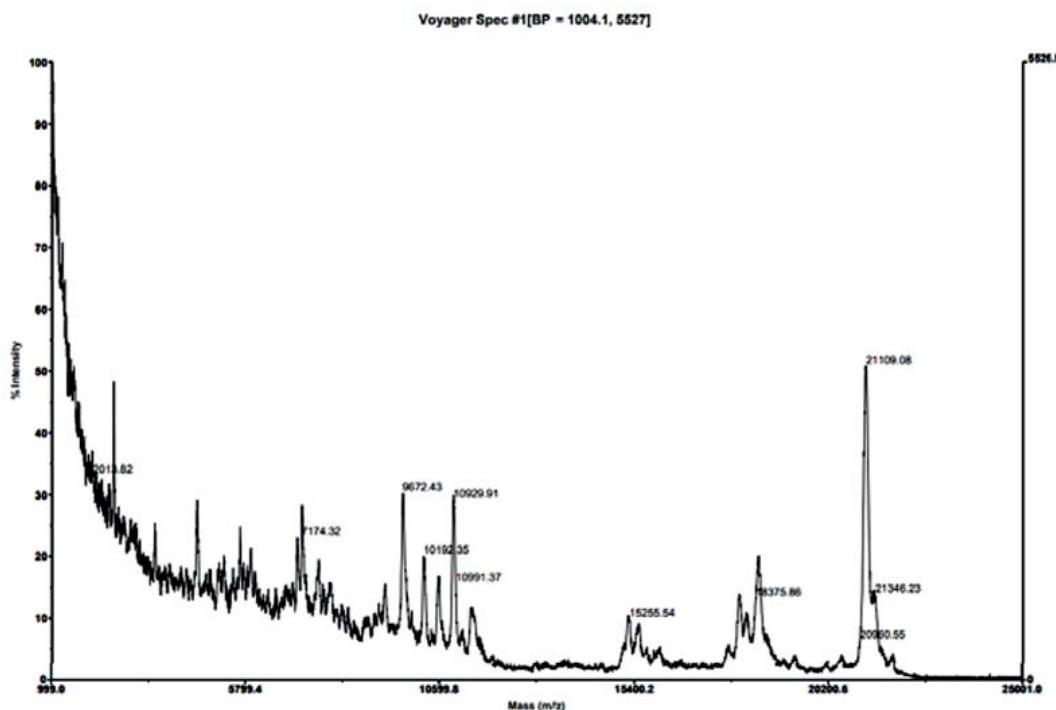


Fig. 4. MALDI-TOF spectrum of hydrolysis products formed by incubation of fibrinogen with proteinase from the venom of *G. halys halys*

The arrows indicate the peaks with a mass to charge ratio of 21109 and 18375

The sequence of the fibrinogen molecule A α 221-610 belongs to the α C-region. In this region, the connector sequence (A α 221-391) and α C-domain (A α 392-610) are distinguished [28]. It is known that α C= domains are involved in intermolecular interactions in the lateral association of protofibrils and fibrils [29]. Also, this sequence is involved in the processes of platelet aggregation [30], migration and proliferation of endothelial and other cells [31, 32].

Our results show that proteinase from the poison of *G. halys halys* cleaves the A α 413-414 bond. Therefore, we can obtain a fragment of fibrinogen A α 414-610 and a form of fibrinogen desA α 414-610 with the help of this proteinase.

This fibrinogen completely preserves the connector sequence of the α C-region, as well as the sequence A α 392-413 of the α C-domain. Study of the peculiarities of the functioning of the partially hydrolyzed form of fibrinogen and the influence of the fragment A α 414-610 on the functioning of native fibrinogen can reveal the role of A α 221-413 and A α 414-610 α C-region in the functioning of fibrinogen.

The proteinase of *G. halys halys* is specific for the A α Lys413-Leu414 peptide bond. Using this enzyme, a partially hydrolyzed form of fibrinogen desA α 414-610 can be obtained from the native fibrinogen of human blood plasma to study the functional role of individual sites of the α C region of fibrinogen.

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СПРЯМОВАНИЙ ПРОТЕОЛІЗ ФІБРИНОГЕНУ ПРОТЕЇНАЗОЮ З ОТРУТИ *Glydius halys halys*

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Мета. Одним із підходів до вивчення структури та функції протеїнів є їх обмежений протеоліз. Отримані шляхом гідролізу фрагменти протеїнів також можуть мати біологічну активність, що може бути використано в дослідженні структурно-функціональних особливостей цих макромолекул. Тому актуальним є пошук селективних протеїназ та визначення специфічності їхньої дії. В цій роботі досліджувалася дія протеїнази з отрути *Glydius halys halys* (щитомордника звичайного) на фібриноген — основний протеїн системи згортання крові.

Методи. Продукти гідролізу фібриногену протеїназою отрути *G. halys halys* досліджували за допомогою методів електрофорезу у поліакриламідному гелі та вестерн-блоту з використанням моноклональних антитіл П-5С (анти-А α 20-78) та 1-5А (анти-А α 549-610). Молекулярну масу продуктів гідролізу визначали за допомогою MALDI-TOF мас-спектрометрії на Voyager DE PRO (США). Послідовності, які відщеплюються від фібриногену досліджуваною протеїназою, встановлювали за допомогою програмного забезпечення «PeptideMassCalculator».

Результати. Електрофоретичний аналіз показав, що протеїназа з отрути *G. halys halys* найбільш специфічно розщеплює А α -ланцюг молекули фібриногену. За допомогою вестерн-блот аналізу було виявлено, що протеїназа відщеплює фрагмент з молекулярною масою приблизно 22 кДа від С-кінця А α -ланцюга молекули фібриногену. Відщеплюваний фрагмент було ідентифіковано MALDI-TOF аналізом як поліпептид з масою 21,1 кДа. Згідно програми «PeptideMassCalculator», цей фрагмент відповідає послідовності А α 414-610. Отже, протеїназа, виділена з отрути *G. halys halys*, специфічно гідролізує пептидний зв'язок А α K413-L414 з утворенням стабільної частково гідролізованої форми — фібриногену desА α 414-610.

Висновок. Використовуючи досліджувану протеїназу, виділену з отрути *G. halys halys*, можна отримати унікальний частково гідролізований фрагмент фібриногену, позбавлений ділянки А α 414-610, яка дозволить дослідити структурно-функціональні особливості α С-регіонів фібрин(оген)у.

Ключові слова: спрямований протеоліз, фібриноген, протеїназа, полімеризація фібрину, гемостаз.