UDC 577.151.6 https://doi.org/10.15407/biotech14.06.037

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

Ye. M. Stohnii
A. V. Rebriev
O. V. Hornytska
O. Yu. Slominskiy
O. P. Kostiuchenko
K. P. Klymenko
V. O. Chernyshenko

Palladin Institute of Biochemistry of NAS of Ukraine, Kyiv, Ukraine

E-mail: stogniyevgen@gmail.com

Received 24.09.2021
Revised 18.11.2021
Accepted 30.12.2021

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 Cand 1-5A targeted to 20–78 and 549–610 fragments of fibrinogen Aα-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" soft ware.

Results. SDS-PAGE showed that protease from the venom of *Gloydius halys halys* initially cleaved Aα-chain of fibrinogen molecule. Western-blot analysis confirmed that this protease specifically cleaves off fragment of C-terminal parts of Aα-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 Aα414-610 residue of fibrinogen molecule. Thus, we showed that studied protease cleaved peptide bond AαK413-L414 with the formation of stable partly hydrolyzed fibrinogen desAα414-610.

Conclusions. The use of protease from the venom of *Gloydius halys halys* would allow obtaining the unique partly hydrolyzed fibrinogen des Aα 414–610 that is suitable for the study of structure and functions of fibrinogen αC-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 Aα 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 *Agkistrodon*, *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 reddishper oxidase (Sigma, USA) were used. Mouse monoclonal antibodies II-5C (anti-Aα20-78) and 1-5A (anti-Aα537-595) 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/ Westernblot

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-Aα20-78) or 1-5A (anti-Aα537-595) for 2 hours, and after washingin TBS, incubated with the secondary secondary anti-mouse goat antibodies labeled with peroxidase. The strips were incubatedin a solution of 0.001 M 4-chloro-1-naphthol in 0.5 M Tris pH 7.5 and 0.03% H_2O_2 to develop specific immunostaining.

Obtaining low molecular weight products of hydrolysis

To obtain a low molecular weight productsof 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% Na_2SO_4. After centrifugation at 3 000 rpm for 30 min, the supernatant containing the enzyme and the cleaved polypeptide was collected and examinedusing 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 Aα chain of fibrinogen, which was hydrolyzed within 90 min. No cleavage of Bβ- and γ-chains was observed during the incubation period.The reaction revealed the accumulation of hydrolysis products of the Aα-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 Aα-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 Aα chain of the fibrinogen molecule (Fig. 2). When using antibody1-5A to the C-terminus of the Aα-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 Aα-chain of fibrinogen.

Detection of two products by Western blot analysis using antibody 1-5A to the C-terminal region of the Aα-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 Aα-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 Aα414-610 and Aα414-583, which are formed by hydrolysis of the AαLys413-Leu414 bond of the fibrinogen molecule.

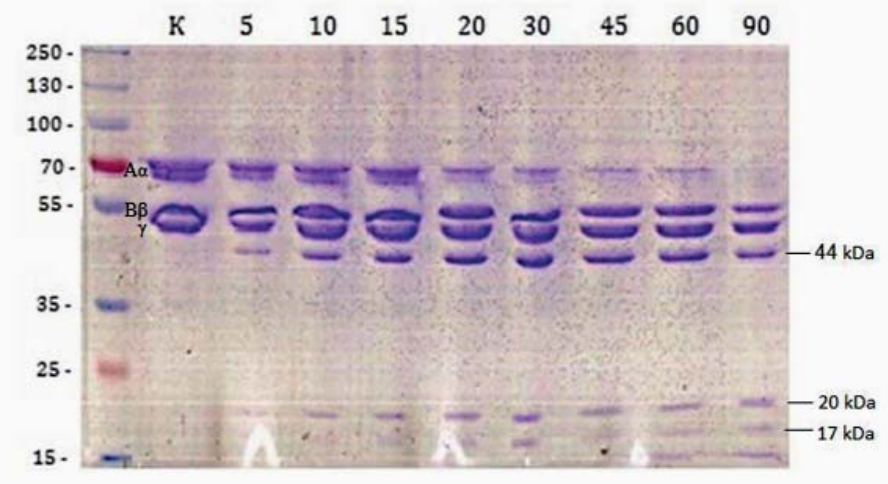

Fig. 1. **Electrophoregram of fibrinogen hydrolysis products by proteinase from the venom of *G. halys halys*:**
K — native fibrinogen; 5, 10, 15, 20, 30, 45, 60, 90 minutes of exposure to the enzyme. The samples were prepared in the presence of 0.2% β-mercaptoethanol. The gel was stained with Coomassie blue

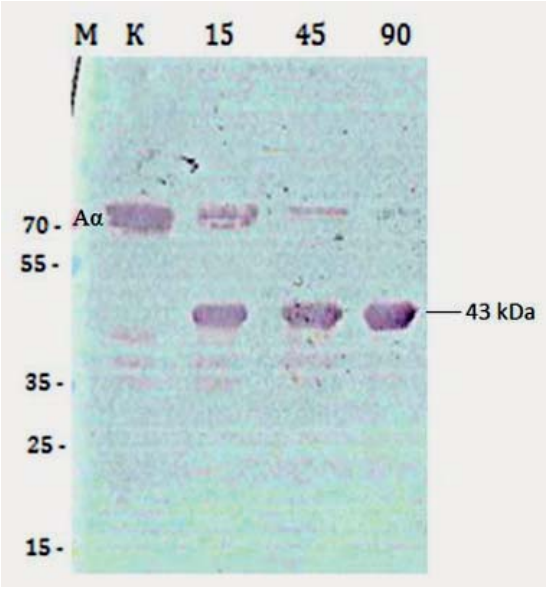

Fig. 2. **Western blot analysis of products of fibrinogen hydrolysis by proteinase from the venom of *G. halys halys* using monoclonal antibody II-5C to the N-terminal regions of the Aα–chain of fibrinogen:**
K — native fibrinogen; 15, 45, 90 minutes of exposure to the enzyme. Samples were prepared in the presence of 0.2% β-mercaptoethanol

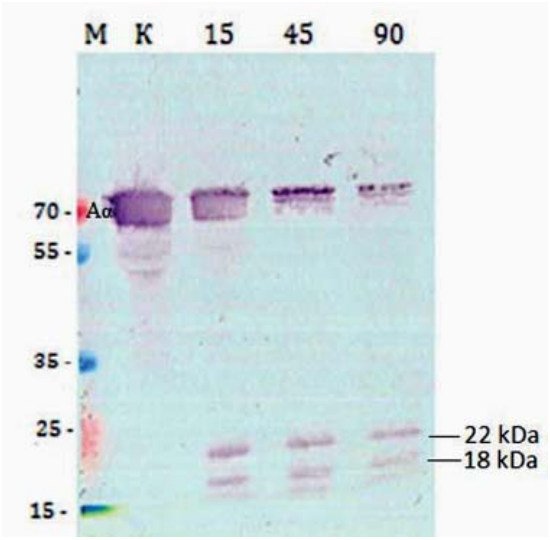

Fig. 3. **Western blot analysis of products of fibrinogen hydrolysis by proteinase from the venom of *G. halys halys* using monoclonal antibodyI-5A to the C-terminal regions of the Aα-chain of fibrinogen:**
K — native fibrinogen; 15, 45, 90 min of exposure to the enzyme. Samples were prepared in the presence of 0.2% β-mercaptoethanol

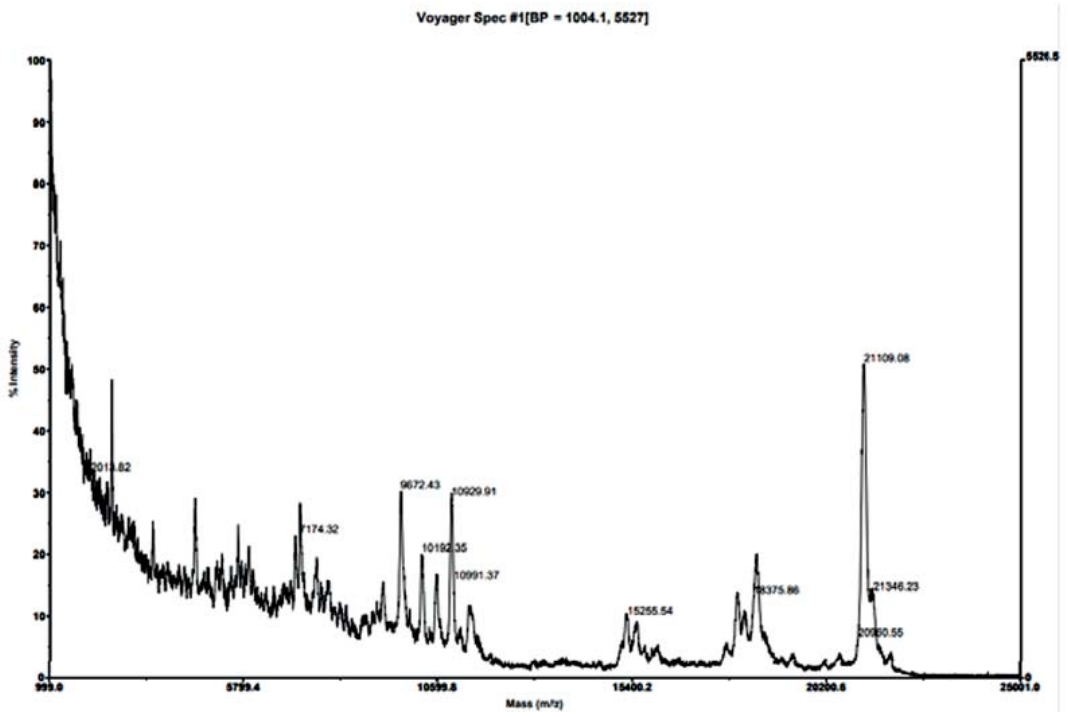

Fig. 4. **MALDI-TOF spectrum of hydrolysis products formed by incubation of fibrinogen with proteinase from the poison of *G. halys halys***
The arrows indicate the peaks with a mass to charge ratio of 21109 and 18375

The sequence of the fibrinogen molecule Aα221-610 belongs to the αC-region. In this region, the connector sequence (Aα221-391) and αC-domain (Aα392-610) are distinguished [28]. It is known that αC = 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 Aα413-414 bond. Therefore, we can obtain a fragment of fibrinogen Aα414-610 and a form of fibrinogen desAα414-610 with the help of this proteinase. This fibrinogen completely preserves the connector sequence of the αC-region, as well as the sequence Aα392-413 of the αC-domain. Study of the peculiarities of the functioning of the partially hydrolyzed form of fibrinogen and the influence of the fragment Aα414-610 on the functioning of native fibrinogen can reveal the role of Aα221-413 and Aα414-610 αC-region in the functioning of fibrinogen.

The proteinase of *G. halys halys* is specific for the AαLys413-Leu414 peptide bond. Using this enzyme, a partially hydrolyzed form of fibrinogen desAα414-610 can be obtained from the native fibrinogen of human blood plasma to study the functional role of individual sites of the αC region of fibrinogen.

REFERENCES

1. *Stohnii Y. M., Ryzhykova M. V., Rebriev A. V., Kuchma M. D., Marunych R. Y., Chernyshenko V. O., Shablii V. A., Lypova N. M., Slominskyi O. Yu., Garmanchuk L. V., Platonova T. M., Komisarenko S. V.* Aggregation of platelets, proliferation of endothelial cell sandmotility of cancer cell sare mediated by the Bβ1(15)-42 residue of fibrin (ogen). *Ukr. Biochem. J.* 2020, 92 (2), 72–84. https://doi.org/10.15407/ubj92.02.072
2. *Kołodziejczyk J., Ponczek M. B.* Therole of fibrinogen, fibrin and fibrin (ogen) degradation products (FDPs) intumor progression. *Contemp. Oncol. (Pozn).* 2013, 17 (2), 113–119. https://doi.org/10.5114/wo.2013.34611
3. *Frangieh J., Rima M., Fajloun Z., Henrion D., Sabatier J.-M., Legros C., Mattei C.* Snake Venom Components: Tools and Cures to Target Cardiovascular Diseases. *Molecules*. 2021, 26 (8), 2223. https://doi.org/10.3390/molecules26082223
4. *Kai Huang, Wei Zhao, Yongxiang Gao, Wenqing Wei, Maikun Teng, Liwen Niu*. Structure of saxthrombin, a thrombin-likeenzyme from *Gloydius saxatilis*. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* 2011, 67 (8), 862–865. https://doi.org/10.1107/S1744309111022548
5. *Cortelazzo A., Guerranti R., L., Hope-Onyekwere N., Muzzi C., Leoncini R., Pagani R.* Effects of snake venom proteases on human fibrinogen chains. *Blood Transfus*. 2010, 8 (3), 120–125. https://doi.org/10.2450/2010.019S
6. *Kini R. M.* Anticoagulant proteins from snake venoms: structure, function and mechanism. *Biochem. J.* 2006, 397 (Pt. 3), 377–387. https://doi.org/10.1042/BJ20060302
7. *Lu Q., Clemetson J. M., Clemetson K. J.* Snake venoms and hemostasis. *J. Thromb. Haemost.* 2005, V. 3, P. 1791–1799. https://doi.org/10.1016/s0041-0101(98)00126-3
8. *Hornytskaia O. V., Platonova T. N., Volkov H. L.* Enzymi zmeynikh yadov. *Ukr. byokhym. zh.* 2003, 75 (3), 22–32.
9. *Xiu-Xia Liang, Ying-Na Zhou, Jia-Shu Chen, Peng-Xin Qiu, Hui-Zhen Chen, Huan-Huan Sun, Yu-Ping Wu, Guang-Mei Yan*. Enzymological Characterization of FII (a), a Fibrinolytic Enzyme From Agkistrodon Acutus Venom. *Acta Pharmacol. Sin*. 2005, 26 (12), 1474–1478. https://doi.org/10.1111/j.1745-7254.2005.00204.x
10. *Meléndez-Martínez D., Plenge-Tellechea L. F., Gatica-Colima A., Cruz-Pérez M. S., Aguilar-Yáñez J. M., Licona-Cassani C.* Functional Mining of the *Crotalus* Spp. Venom Protease Repertoire Reveals Potential for Chronic Wound Therapeutics. *Molecules*. 2020, 25 (15), 3401. https://doi.org/10.3390/molecules25153401
11. *Choo Hock Tan, Kae Yi Tan, Tzu Shan Ng, Evan S. H. Quah, Ahmad Khaldun Ismail, Sumana Khomvilai, Visith Sitprija, Nget Hong Tan*. Venomics of *Trimeresurus (Popeia) nebularis*, the Cameron Highlands Pit Viper from Malaysia: Insights into Venom Proteome, Toxicity and Neutralization of Antivenom. *Toxins (Basel)*. 2019, 11 (2), 95. https://doi.org/10.3390/toxins11020095
12. *Huang J., Fan H., Yin X., Huang F.* Isolation of a Novel Metalloproteinase from Agkistrodon Venom and Its Antithrombotic Activity Analysis. *Int. J. Mol. Sci.* 2019, 20 (17), 4088. https://doi.org/10.3390/ijms20174088
13. *Senji Laxme R. R., Attarde S., Khochare S., Suranse V., Martin G., Casewell N. R., Whitaker R., Sunagar K.* Biogeographical venom variation in the Indian spectacled cobra (*Najanaja*) underscores the pressing

need for pan-India efficacious snakebite therapy. *PLoS Negl. Trop. Dis.* 2021, 15 (2), e0009150. https://doi.org/10.1371/journal.pntd.0009150

14. *Yong-Hong Jia, Yang Jin, Qiu-Min Lü, Dong-Sheng Li, Wan-Yu Wang, Yu-Liang Xiong*. Jerdonase a novel serine protease with kinin-releasing and fibrinogenolytic activity from *Trimeresurusjerdonii* venom. *Sheng wuhuaxueyu sheng wuwu li xuebao Acta Biochimica et Biophysica Sinica*.2003, V. 35, P 689–694.
15. *De-Simone S. G., Correa-Netto C., Antunes O. A. C., Alencastro R. B. De, Silva Jr. F. P.* Biochemical and molecular modeling analysis of the ability of two p-aminobenzamidine-based sorbents to selectively purify serine proteases (fibrinogenases) from snake venoms. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2005, 822 (1–2), 1–9. https://doi.org/10.1016/j.jchromb.2005.04.018
16. S*uk-Ho Choi, Seung-Bae Lee.* Isolation from Gloydius blomhoffii siniticus Venom of a Fibrin(ogen)olytic Enzyme Consisting of Two Heterogenous Polypeptides. *J. Pharmacopuncture.* 2013, 16 (2), 46–54. https://doi.org/10.3831/KPI.2013.16.010
17. *He J., Chen S., Gu J.* Identification and characterization of Harobin, anovel fibrino(geno)lytic serine protease from a sea snake (*Lapemis hardwickii*). *FEBS Lett*. 2007, 581 (16), 2965–2973. https://doi.org/10.1016/j.febslet.2007.05.047
18. *Tarek Mohamed Abd El-Aziz, Antonio Garcia Soares, James D. Stockand*. Snake Venoms in Drug Discovery: Valuable Therapeutic Tools for Life Saving. *Toxins (Basel)*. 2019, 11 (10), 564. https://doi.org/10.3390/toxins11100564
19. *Lu Q., Clemetson J. M., Clemetson K. J.* Snake venoms and hemostasis. *J. Thromb. Haemost.* 2005, V. 3, P. 1791–1799. https://doi.org/10.1111/j.1538-7836.2005.01358.x
20. *Ghorbanpur M., Zare A., Mirakabadi F., Zokaee H., Zolfagarrian H. Rabiei*. Purification and partial characterization of a coagulant serine protease from the venom of the Iranian snake Agkistrodon halys. *J. Venom. Anim. Toxinsincl. Trop.* 2009, 15 (3). https://doi.org/10.1590/S1678-91992009000300005
21. *Gornitskaia O. V., Rovinskaya I. N., Platonova T. N.* Purification and characterization of the fibrinolytic enzyme from Agkistrodon halys halys venom. *Ukr. Biokhim. Zh. (1999)*. 2002, 74 (3), 42–49. (Russian). PMID: 12916236
22. *Varetskaia V.* Preparation of a fibrin monomer and studies on some of its properties. *Ukr. Biokhim. Zh.* 1965, 37 (2), 194–206.
23. *Urvant L.P., Makogonenko E.M., Pozniak T.A., Pydiura N. A., Kolesnikova I. N., Tsap P. Y., Bereznitzkiy G. K., Lugovskoy E. V., Komisarenko S. V.* Binding of mAb II-5c to Aα20–78 fragment of fibrinogen inhibits a neoantigenic determinant exposure within Bβ126–135 site of a molecule. *Reports of NAS*. 2014, V. 5, P. 149–156. https://doi.org/10.15407/dopovidi2014.05.149
24. *Lugovska N.E., Kolesnikova I.M., Stohnii Ye.M., Chernyshenko V. O., Rebriev A. V., Kostiuchenko O. P., Gogolinska G. K., Dziubliuk N.A., Varbanets L. D., Platonova T. M., Komisarenko S. V.* Novel monoclonal antibody to fibrin(ogen) αC-region for detection of the earliest forms of soluble fibrin. *Ukr. Biochem. J.* 2020, 92 (3), 58–70. https://doi.org/10.15407/ubj92.03.058
25. *Laemli R. V.* Cleavage of structural poteins during of bacteriophage T4. *Nature.* 1970, V. 227, P. 680–685. https://doi.org/10.1038/227680a0
26. *Chapman J. R.* Mass Spectrometry of Proteins and Peptides. *Methods in Molecular Biology*. 2000, V. 146, P. 554.
27. *Lugovskoi E. V., Makogonenko E. M., Komisarenko S. V.* Molecular mechanisms of formation and degradation of fibrin. *Kyiv: Naukova dumka*. 2013, 230p.
28. *Tsurupa G., Mahid A., Veklich Y., Weisel J. W., Medved L.* Structure, Stability, and Interaction of Fibrin αC-Domain Polymers. *Biochemistry*. 2011, V. 50, P. 8028–8037. https://doi.org/10.1021/bi2008189
29. *John W. Weisel, Rustem I. Litvinov*. Fibrin Formation, Structure and Properties. Subcell Biochem. 2017, V. 82, P. 405–456. https://doi.org/10.1007/978-3-319-49674-0_13
30. *Wencel-Drake J. D., Boudignon-Proudhon C., Dieter M. G., Criss A. B, Leslie V. Parise*. Internalization of bound fibrinogen modulates platelet aggregation. *Blood.* 1996, 87 (2), 602–612.
31. *Yakovlev S., Mikhailenko I., Tsurupa G., Belkin A. M., Medved L.* Polymerization of fibrin αC-domains promotes endothelial cell migration and proliferation. *Thromb. Haemost*. 2014, 112 (6), 1244–1251. https://doi.org/10.1160/TH14-01-0079
32. *Tsurupa G., Hantgan R. R., Burton R. A., Pechik I., Tjandra N., Medved L.* Structure, Stability, and Interaction of the Fibrin(ogen) αC-Domains. *Biochemistry*. 2009, 48 (51), 12191–12201. https://doi.org/10.1021/bi901640e

СПРЯМОВАНИЙ ПРОТЕОЛІЗ ФІБРИНОГЕНУ ПРОТЕЇНАЗОЮ З ОТРУТИ *Gloydius halys halys*

Є. М. Стогній, А. В. Ребрієв, О. В. Горницька, О. Ю. Сломінський, О. П. Костюченко, К. П. Клименко, В. О. Чернишенко

Інститут біохімії ім. О. В. Палладіна НАН України, Київ

E-mail: stogniyevgen@gmail.com

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 *Gloydius 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 II-5C (анти-Aα20-78) та 1-5A (анти-Aα549-610). 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 Aα-ланцюг молекули фібриногену. За допомогою вестерн-блот аналізу було виявлено, що протеїназа відщеплює фрагмент з молекулярною масою приблизно 22 кDa від С-кінця Aα-ланцюга молекули фібриногену. Відщеплюваний фрагмент було ідентифіковано MALDI-TOF аналізом як поліпептид з масою 21,1 кDa. Згідно програми «PeptideMassCalculator», цей фрагмент відповідає послідовності Aα414-610. Отже, протеїназа, виділена з отрути *G. halys halys*, специфічно гідролізує пептидний зв'язок AαK413-L414 з утворенням стабільної частково гідролізованої форми — фібриногену desAα414-610.

Висновок. Використовуючи досліджувану протеїназу, виділену з отрути *G. halys halys*, можна отримати унікальний частково гідролізований фрагмент фібриногену, позбавлений ділянки Aα414-610, яка дозволить дослідити структурно-функціональні особливості αC-регіонів фібрин(оген)у.

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

12. *Daradka H. M., Abas M. M., Mohammad M.* Antidiabetic effect of *Artemisia absinthium* extracts on alloxan-induced diabetic rats. *Comp. Clin. Path.* 2014, 23 (6), 1733–1742. https://doi.org/10.1007/s00580-014-1963-1
13. *Turak A., Shi S.-P., Jiang Y., Tu P. F.* Dimeric guaianolides from *Artemisia absinthium*. *Phytochemistry*. 2014, V. 105, P. 109–114. https://doi.org/10.1016/j.phytochem.2014.06.016
14. *Pietta P. G.* Flavonoids as antioxidants. *J. Nat. Prod*. 2000, 63 (7), 1035–1042. https://doi.org/10.1021/np9904509
15. *Pisoschi A. M., Pop A.* The role of antioxidants in the chemistry of oxidative stress: A review. *Eur. J. Med. Chem*. 2015, V. 97, P. 55–74. https://doi.org/10.1016/j.ejmech.2015.04.040
16. *Giri A., Narasu M. L.* Transgenic hairy roots: recent trends and applications. *Biotechnol. Adv*. 2000, V. 18, P. 1–22. https://doi.org/10.1016/S0734-9750(99)00016-6
17. *Bulgakov V. P.* Functions of rol genes in plant secondary metabolism. *Biotechnol. Adv*. 2008, V. 26, P. 318–324. https://doi.org/10.1016/j.biotechadv.2008.03.001
18. *Balasubramanian M., Anbumegala M., Surendran R., Run M., Shanmugam G.* Elite hairy roots of *Raphanus sativus* (L.) as a source of antioxidants and flavonoids. *3 Biotech*. 2018, V. 8, P. 128. https://doi.org/10.1007/s13205-018-1153-y
19. *Ono N. N., Tian L.* The multiplicity of hairy root cultures: prolific possibilities. *Plant Sci*. 2011, 180 (3), 439–446. https://doi.org/10.1016/j.plantsci.2010.11.012
20. *Chandra S.* Natural plant genetic engineer *Agrobacterium* rhizogenes: role of T-DNA in plant secondary metabolism. *Biotechnol. Lett*. 2012, 34 (3), 407–415. https://doi.org/10.1007/s10529-011-0785-3
21. *Kim Y., Wyslouzil B. E., Weathers P. J.* Secondary metabolism of hairy root cultures in bioreactors. *In Vitro Cell. Dev. Biol.-Plant*. 2002, 38 (1), 1–10. www.jstor.org/stable/20171597
22. *Abraham J., Thomas T. D.* Hairy Root Culture for the Production of Useful Secondary Metabolites. *Biotechnology and Production of Anti-Cancer Compounds*. 2017, P. 201–230. https://doi.org/10.1007/978-3-319-53880-8_9
23. *Balasubramani S., Ranjitha Kumari B. D., Moola A. K., Sathish D., Prem Kumar G., Srimurali S., Babu Rajendran R.* Enhanced Production of β-Caryophyllene by Farnesyl Diphosphate Precursor-Treated Callus and Hairy Root Cultures of *Artemisia vulgaris* L. *Front. Plant Sci*. 2021, V. 12, P. 634178. https://doi.org/10.3389/fpls.2021.634178
24. *Zheng L. P., Guo Y. T., Wang J. W., Tan R. X.* Nitric oxide potentiates oligosaccharide-induced artemisinin production in *Artemisia annua* hairy roots. *J. Integr. Plant Biol*. 2008, 50 (1), 49–55. https://doi.org/10.1111/j.1744-7909.2007.00589.x
25. *Pala Z., Shukla V., Alok A., Kudale S., Desai N.* Enhanced production of an anti-malarial compound artesunate by hairy root cultures and phytochemical analysis of *Artemisia pallens* Wall. *3 Biotech*. 2016, 6 (2), 182. https://doi.org/10.1007/s13205-016-0496-5
26. *Nin S., Bennici A., Roselli G., Mariotti D., Schiff S., Magherini R.* Agrobacterium-mediated transformation of *Artemisia absinthium* L. (wormwood) and production of secondary metabolites. *Plant Cell Reports*. 1997, 16 (10), 725–730. https://doi.org/10.1007/s002990050310. PMID: 30727627
27. *Leth I. K., McDonald K. A.* Media development for large scale *Agrobacterium tumefaciens* culture. *Biotechnology Progress*. 2017, 33 (5), 1218–1225. https://doi.org/10.1002/btpr.2504
28. *Aboul-Maaty N. A. F., Oraby H. A. S.* Extraction of high-quality genomic DNA from different plant orders applying a modified CTAB-based method. *Bull. Nat. Res. Centre*. 2019, 43 (25). https://doi.org/10.1186/s42269-019-0066-1
29. *Singleton V. L, Orthofer R., Lamuela-Raventós R. M.* Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods in Enzymology, Academic Press*. 1999, V. 299, P. 152–178. https://doi.org/10.1016/S0076-6879(99)99017-1
30. *Pękal A., Pyrzynska K.* Evaluation of Aluminium Complexation Reaction for Flavonoid Content Assay. *Food Anal. Methods*. 2014, V. 7, P. 1776–1782. https://doi.org/10.1007/s12161-014-9814-x
31. *Brand-Williams W., Cuvelier M. E., Berset C.* Use of a free radical method to evaluate antioxidant activity. *LWT — Food Science and Technology*. 1995, 28 (1), 25–30. https://doi.org/10.1016/S0023-6438(95)80008-5
32. *Tavassoli P., Safipour Afshar A.* Influence of different *Agrobacterium rhizogenes* strains on hairy root induction and analysis of phenolic and flavonoid compounds in marshmallow (*Althaea officinalis* L.). *3 Biotech*. 2018, 8 (8), 351. https://doi.org/10.1007/s13205-018-1375-z
33. *Sahayarayan J. J., Udayakumar R., Arun M., Ganapathi A., Alwahibi M. S., Aldosari N. S., Morgan A.* Effect of different *Agrobacterium rhizogenes* strains for *in vitro* hairy root induction, total phenolic, flavonoids

contents, antibacterial and antioxidant activity of (*Cucumis anguria* L.). *Saudi J. Biol. Sci.* 2020, 27 (11), 2972–2979. https://doi.org/10.1016/j.sjbs.2020.08.050
34. *El-Esawi M. A., Elkelish A., Elansary H. O., Ali H. M., Elshikh M., Witczak J., Ahmad M.* Genetic Transformation and Hairy Root Induction Enhance the Antioxidant Potential of *Lactuca serriola* L. *Oxid. Med. Cell. Longev.* 2017, V. 2017, P. 5604746. https://doi.org/10.1155/2017/5604746

Agrobacterium rhizogenes — ОПОСЕРЕДКОВАНА ТРАНСФОРМАЦІЯ ЯК СПОСІБ СТИМУЛЮВАННЯ СИНТЕЗУ АНТИОКСИДАНТНИХ СПОЛУК У *Artemisia absinthium* L.

А. І. Ольховська, К. О. Дробот, А. М. Шаховський, Н. А. Матвєєва

Інститут клітинної біології та генетичної інженерії НАН України, Київ

E-mail: nolkhovskaya2012@gmail.com

Рослини *Artemisia absinthium* L. відомі як продуценти речовин з антиоксидантними властивостями. Зокрема, в них виявлено поліфеноли та флавоноїди. Активізувати синтез цих сполук можна шляхом генетичної трансформації навіть без перенесення специфічних генів, які беруть участь у біосинтезі. Так, «бородаті» корені, одержані після *Agrobacterium rhizogenes*-опосередкованої трансформації, можуть бути продуцентами комплексу цінних метаболітів.

Метою роботи було одержати «бородаті» корені *A. absinthium* як продуценти поліфенольних сполук.

Методи. «Бородаті» корені одержували шляхом культивування листків з суспензією *A. rhizogenes* з вектором *pCB124*. Наявність перенесених генів підтверджували методом ПЛР. Для визначення вмісту флавоноїдів та поліфенолів використовували реакції з $AlCl_3$ та реактивом Фоліна-Чокальте. Антиоксидантну активність оцінювали за здатністю екстрактів відновлювати DPPH радикал.

Результати. ПЛР аналіз виявив наявність бактеріальних *rol* генів та відсутність генів плазміди pCB124. Лінії коренів відрізнялися між собою за швидкістю росту. «Бородаті» корені характеризувалися більшим вмістом поліфенолів, зокрема, флавоноїдів (до 4.784±0.10 мг/г ВМ) та вищим рівнем антиоксидантної активності (EC_{50} = 3.657 мг) у порівнянні з контролем (3.861±0,13 мг/г СМ та EC_{50} = 6.716 мг відповідно).

Висновки. Трансформацію *A. absinthium* із застосуванням *A. rhizogenes* може бути використано для одержання ліній з підвищеним вмістом поліфенольних сполук та більшою антиоксидантною активністю.

Ключові слова: Artemisia absinthium L., *Agrobacterium rhizogenes*-опосередкована трансформація, «бородаті» корені, флавоноїди, поліфенольні сполуки, антиоксидантна активність.