UDC 57.088.3 **https://doi.org/10.15407/biotech15.06.061**

STANDARDIZATION OF THE PROTEIN CALIBRATORS ISOLATION METHODOLOGY FOR THROMBOPHILIA MARKERS DETECTING IMMUNODIAGNOSTIC TEST SYSTEMS

Daria Korolova[1]
Myroslav Syrko[1, 2]
Yevhenii Stohnii[1]
Nadiia Druzhyna[1]
Tamara Chernyshenko[1]
Genrietta Gogolinska[1]

[1]Palladin Institute of Biochemistry of the National Academy of Sciences, Kyiv, Ukraine

[2]Taras Shevchenko National University of Kyiv, Ukraine

E-mail: myroslaav@gmail.com

Received 11.10.2022
Revised 14.12.2022
Accepted 30.12.2022

The most accurate laboratory methods for thrombophilia diagnostics are based on the quantitative determination of the blood plasma specific markers that appear as a result of the coagulation cascade activation. Soluble fibrin and D-dimer belong to the main of the last ones. An alteration in the concentration of such markers can indicate thrombin concentration growth and the formation of soluble oligomeric fibrin. It should be pointed out that simultaneous detection of these markers can establish the correlation between the accumulation of soluble fibrin and fibrinolysis and nowadays is provided only by enzyme-linked immunoassay. Thus, the usage of immunodiagnostic test systems for the detection of thrombophilia markers is highly relevant today. The important components of immunodiagnostic test system are protein calibrators, the isolation standardization of which plays a key role for accurate construction of a calibration curve and obtaining objective results as a consequence.

The objective of this study was to develop the soluble fibrin and D-dimer isolation methodology and its standardization for their further use as the protein calibrators for thrombophilia markers detecting immunodiagnostic test systems.

Materials and Methods. Soluble fibrin and D-dimer were isolated from collected human blood by fibrinogen salting out with further fibrin polymerization with thrombin and hydrolysis with plasmin. Quality control of the obtained proteins was carried out using SDS-PAGE and turbidimetric measurements with further checking of the proteins as calibrators for the thrombophilia markers detecting immunoassay.

Results. Obtained proteins meet the necessary specifications and can be used as calibrators for immunodiagnostic test systems. Soluble fibrin and D-dimer were checked by SDS-PAGE for the absence of impurities. Turbidimetric measurements showed the polymerization capability of the soluble fibrin and the inhibition of the polymerization by D-dimer.

Conclusion. The standardized isolation methodology of soluble fibrin and D-dimer can be used to obtain protein calibrators for appropriate immunodiagnostic test systems.

Key words: protein calibrators; thrombophilia; soluble fibrin; D-dimer; immunodiagnostic test systems.

Highly accurate laboratory diagnostics of the hemostasis system state, as well as the possibility of timely prediction of potential thrombotic complications, remain a critically demanding problem in the modern medicine [1]. Intravascular thrombus formations of various nature and localization frequently cause death or disability, notably in cardiovascular, autoimmune, oncological and number of other pathologies. Simultaneously, an increase in the number of cases of intravascular thrombosis of various localization has been reported in recent years, as evidenced by the data of clinical practice [2–4]. Therefore, the development and

improvement of the thrombophilia markers detecting methods are the essential tools for the opportune detection of the threat of thrombosis. Modern laboratory diagnostics of the hemostasis system state should ensure not only the timely detection of the intravascular blood clot formation threat, but also help in the effectiveness monitoring of antithrombotic therapy [5].

Nowadays a number of laboratory methods for intravascular thrombus formation diagnosing have been developed and implemented in diagnostic practice. However, most of these methods are characterized by low sensitivity and informativeness for an accurate and reliable understanding of the real hemostasis system state. Modern screening tests commonly do not allow predicting the pathological intravascular thrombosis development and subsequent complications, since they do not satisfy all the necessary requirements for laboratory diagnostics of thrombophilia. Accordingly, the possibility of early antithrombotic treatment planning and its monitoring is complicated. The main screening tests that are widely used today to diagnose the hemostasis system state include the activated partial thromboplastin time test, prothrombin and thrombin time tests, platelet aggregation test, as well as fibrinogen concentration determination by the weight of the formed fibrin clot or the time spent on fibrin polymerization [6,7]. Such methods can only reflect the state of particular hemostasis links or functioning of the coagulation cascade particular factors, fibrinolytic potential, individual processes related to the functional properties of platelets, etc. As opposed to that methodologies, comprehensive thrombophilia diagnostics should include a number of indicators that, on the one hand, point to specific markers of coagulation system activation, and on the other hand, allow to understand the development of specific pathological mechanisms [1].

The most accurate and informative methods of thrombophilia diagnostics are based on the quantitative determination of the specific markers of the blood coagulation system activation, since the thrombosis diagnostics based only on clinical manifestations does not allow the determination of the intravascular coagulation risk. These markers include fibrinogen derivatives, namely D-dimer and soluble fibrin [8, 9]. An alteration in the concentration of the specific blood coagulation markers can indicate an increase in the thrombin concentration in the blood plasma and subsequent cascade processes of the formation of monomeric fibrin from fibrinogen as well as soluble oligomeric fibrin [5].

Soluble fibrin is characterized as a highly specific marker of the hemocoagulation system activation [10]. By chemical nature it is a soluble oligomeric complex of fibrin molecules with fibrinogen that freely circulate in the blood plasma. An increase in the soluble fibrin concentration in the blood plasma indicates the thrombin activity, which converts fibrinogen into monomeric fibrin [11]. Accordingly, the concentration of soluble fibrin, as an early marker of the thrombus formation threat, determines the degree of coagulation activation. Despite the availability of immunoenzymatic test systems for soluble fibrin quantitative determining in blood plasma, semiquantitative methods are usually used in modern clinical practice. In particular, precipitation methods using chemical agents and functional methods based on the property of soluble fibrin to stimulate the formation of plasmin from plasminogen worth highlighting. The main drawback of the listed approaches is the impossibility of accurate determining the concentration of soluble fibrin in blood plasma. Despite the small number of monoclonal antibodies with soluble fibrin specificity suitable for use in test systems, immunodiagnostic methods have a tendency to develop as quantitative data provide an opportunity to objectively assess the risks of intravascular thrombus formation [12,13].

D-dimer is a specific product of plasmin cleavage of stabilized by factor XIIIa fibrin, that indirectly indicates the formation of fibrin and its stabilization, in other words, it can be characterized as a marker of both the formation of stabilized fibrin and fibrinolysis [4]. Laboratory methods for D-dimer determining are one of the most popular diagnostic practices in the study of the coagulation system [14, 15]. The arsenal of D-dimer determining methods includes both qualitative and semi-quantitative methods, in particular the agglutination reaction of latex parts, as well as number of quantitative methods: immunochromatography, immunoturbidimetry, immunodiffusion on porous membranes, along with the enzyme linked immunosorbent method, which allows quantitative determination of the target protein with high sensitivity [16–18]. Quantitative detection of D-dimer is mandatory for the intravascular venous thrombosis diagnostics, in particular for the

deep vein thrombosis or pulmonary embolism [6,19]. It should be pointed out that modern D-dimer immunoenzymatic diagnostics is not standardized, a large number of commercial test systems differ in methods of conducting and usage of different baselines [17].

Since the concentration of thrombophilia markers in the blood plasma and their ratio indicate the correlation between the formation and destruction of fibrin clot, the simultaneous quantitative determination of soluble fibrin and D-dimer allows to detect an imbalance between the systems of blood coagulation and fibrinolysis [5]. In particular, simultaneous slight increase in the soluble fibrin and D-dimer concentration indicates a balance between the coagulation and fibrinolysis systems, while a significant increase in the concentrations of D-dimer can serve as an indicator of the presence of stabilized fibrin clot in the bloodstream, as well as the activation of its lysis. Simultaneously, the change in the concentration of soluble fibrin can be characterized as an important molecular marker of the coagulation system activation degree. In some cases, only simultaneous detection of thrombophilia markers ensure accurate study of the intravascular thrombus formation threat. For example, it plays an important role in postoperative conditions, because in such cases the disorders of the hemostasis system can increase. Consequently, simultaneous quantitative determination of the soluble fibrin and D-dimer concentration is an important diagnostic indicator that must be used in clinical practice. Furthermore, the simultaneous determination of thrombophilia markers nowadays is ensured only by enzyme-linked immunoassay [6].

The main advantages of immunoenzymatic methods for thrombophilia markers determining are high specificity and sensitivity, quantitative automated detection, stability of the immunosorbent during storage and the absence of the interference with pathological inhibitors of the coagulation cascade or heparin preparations in the blood plasma of patients [16, 20]. Diagnostic test systems for the determination of fibrinogen, soluble fibrin and D-dimer were developed by the O.V. Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine based on the principle of a bi-site solid-phase immunoenzymatic technique. This method allows quick determination of the thrombophilia molecular markers with a high degree of accuracy. FnI-3C and III-3B monoclonal antibodies with high specificity to soluble fibrin and D-dimer accordingly are capable of selective target binding and do not interact with other blood proteins, therefore they are used as an immunological sorbent. After binding the last with target proteins, secondary detection II-4d monoclonal antibodies with high specificity are added. To amplify the signal, secondary antibodies are biotinylated and the biotin-streptavidin-peroxidase complex is formed. The concentration of the proteins is determined by the intensity of the color relative to the calibration curve. The last is built using the appropriate protein calibrators [6]. Consequently, the standardization of the soluble fibrin and D-dimer obtaining methodology for their further use as protein calibrators for an accurate construction of the calibration curve was the objective of this study.

The developed test systems for the determination of thrombophilia molecular markers have undergone successful clinical trials for the study of pathologies associated with intravascular thrombus formation, in particular cardiovascular diseases, diabetes, the consequences of surgical intervention, etc. It is important to note that the simultaneous determination of the main blood coagulation markers allows obtaining both detail-oriented and a big picture analysis of the hemostasis system state and enables early diagnostics of prethrombotic conditions, timely and correct antithrombotic therapy selection and treatment effectiveness monitoring [5,6]. Taking into account the high accuracy of soluble fibrin and D-dimer determination using immunoenzymatic methods, the development of appropriate diagnostic test systems and the standardization of their components, in particular protein calibrators, as well as their implementation into clinical practice are important problems for the modern clinical thrombophilia diagnostics.

Materials and Methods

Human blood plasma. Donor blood was obtained at the Main Military Hospital of the Ministry of Defense of Ukraine (Kyiv, Ukraine) from healthy volunteers (25–35 years old) who had not taken any medicines for 7 days. Prior to blood taking informed consent was signed by all volunteers. Immediately after taking into sterile plastic tubes, blood was mixed with an anticoagulant in a ratio of 9:1 and cooled on ice. The aqueous solution of the anticoagulant contained: 3.8% sodium citrate and 0.262%

ε-aminocaproic acid. Plasma was separated by centrifugation (twice at 1200–1400 g, 60 min, 5 °C) of pooled citrate-anticoagulated blood from donors. An aqueous suspension of $BaSO_4$ was added to cooled plasma (with the ratio of 60 g of $BaSO_4$ per 1liter), stirred for 45 min at 4 °C and centrifuged at 1200–1400 g for 10 min to precipitate prothrombin.

Fibrinogen isolation. Human fibrinogen was isolated from blood plasma by fractional sodium sulfate (Na_2SO_4) precipitation. 1 M glycine buffer (pH 9.5) containing 0.1 M NaCl was added to prothrombin-free plasma (in the ratio 1:9) and stirred at 20–22 °C using water bath. During constant stirring 16% Na_2SO_4 solution was added dropwise to plasma (520–680 ml of a 16% Na_2SO_4 per 1 liter of plasma) until the appearance of a fine sediment. The last was removed by centrifugation at 1400 g for 15 min with further fibrinogen precipitation from the supernatant by adding 16% Na_2SO_4 dropwise to its final concentration of 8.5% and centrifugation at 1200 g for 15 min. Then the precipitate was dissolved in 0.2 M NaCI (the sediment from 1 liter of initial plasma in 200 ml of 0.2 M NaCI) and an equal volume of 16% Na_2SO_4 was added again dropwise and centrifuged at 1200 g for 20 min. The precipitate was dissolved in 0.2 M NaCl (the sediment from 1 l of plasma in 200 ml of 0.2 M NaCl). 1/5 of the volume of 0.5 M potassium phosphate buffer (pH 6.5) was added to resulting solution. Fibrinogen was salted out again by adding an equal volume of 16% Na_2SO_4 and centrifuged at 1200 g for 20 min. The precipitate was dissolved in 0.15 M NaCl. The concentration of fibrinogen was adjusted to 1% with 0.15 M NaCl and left for 16 h at +4 °C. Then solution was centrifuged at 1400 g for 15 minutes, while cryofibrinogen remained in the precipitate. An equal volume of 16% Na_2SO_4 was added to the supernatant and centrifuged at 1200 g for 15 min. The precipitate was dissolved in 0.15 M NaCl to a final fibrinogen concentration o/f 25–30 mg/ml. Fibrinogen solution was stored at –20 °C.

desAB fibrin purification. desAB fibrin was obtained by thrombin enzymatic action on fibrinogen. Thrombin (0.15–0.3 NIH per 1 mg of fibrinogen) was added to 0.25% solution of fibrinogen in 0.15 M NaCl and incubated for 1–1.5 h at 37 °C. After incubation, the clot was removed and washed twice by 0.15 M NaCl. The resulting fibrin clot was dissolved in 0.125% acetic acid.14 volumes of 0.063 potassium phosphate buffer with 0.25M NaCl (pH 6.0) was added to fibrin solution and left for 2 h for fibrin polimerization. Resulting clot was washed two times by 0.15M NaCl, the third time by 0.075M NaCl and dissolved in 0.0125% acetic acid at 4 °C. Fibrin solution was centrifuged 10–15 min. at 900 g at 5 °C. This cycle of polimerization and dissolving was repeated twice. Obtained 1.2–1.5% desAB fibrin solution in 0.0125% M acetic acid was stored at 4 °C.

D-dimer purification. D-dimer fragment was obtained from cross-linked fibrin cleaved by plasmin. Conversion of fibrinogen to fibrin followed by its stabilization was performed in 0.05 M Tris-HCI buffer (pH 7.4) containing 0.13 M NaCl, 0.02 M $CaCl_2$, 0.001 M NaN_3 (1 NIH of thrombin per 1 mg of fibrinogen was added). Incubation was carried out for 3 h at 37 °C. Stabilized fibrin was lyzed by plasmin for 6 hours using the concentration of 0.4 caseinolitic units per 1ml of lyzate. Hydrolysis was stopped by adding diisopropylfluorophosphate, after which plasmin was excluded on Lys-sepharose. Isolation of fragments was carried out by affinity chromatography. The mixture was applied to a column with immobilized fibrin monomer in 0.05 M Tris-H_3PO_4 buffer containing 0.1 M NaCl, 0.025 M ε-aminocaproic acid, 10^{-4} M $CaCl_2$ (pH 6.85). D fragment impurities were eluted with 0.05 M Tris-H_3PO_4 buffer containing 0.25 M NaCl, 0.025 M ε-aminocaproic acid and 10^{-4} M $CaCl_2$ (pH 5.9). D-dimer was eluted with 0.05 M Tris-H_3PO_4 buffer containing 1.0 M NaCl, 0.025 M ε-aminocaproic acid and 10^{-4} M $CaCl_2$ (pH 5.3).

Protein concentration identification. Concentration of obtained proteins was identified by measurement of optic density at 280 nm subtracting the absorbency at 320 nm using the spectrophotometer POP (Optizen, Daejeon, Korea).

Electrophoresis in polyacrylamide gel. The purity of obtained protein samples was determined by SDS-PAGE using 7% polyacrylamide gel according to Laemmli with the use of Tris-glycine system. Electrophoresis buffer was prepared using 22.7 g of tris(hydroxymethyl)aminomethane, 0.5 g of SDS, 1 ml of TEMED per 250 ml (pH 8.8). The concentrating buffer was prepared with the ratio of 3.03 g of tris(hydroxymethyl) aminomethane, 0.2 g of SDS, 0.4 ml of TEMED per 100 ml (pH 6.8). The 7% polyacrylamide gel was prepared with 6 ml of electrophoresis buffer, 3.65 ml of distilled water, 2.1 ml of 40% acrylamide solution, 0.25 ml of 1.5% ammonium persulfate. The concentrating gel was prepared with 2 ml of concentrating

buffer, 0.4 ml of 40% acrylamide solution, 1.4 ml of distilled water, 0.2 ml of 1.5% ammonium persulfate. The running buffer solution was prepared with the ratio of 6 g/l tris(hydroxymethyl)aminomethane, 28.8 g/l of glycine, 1 g/l of SDS.

Samples for electrophoresis were prepared by adding 1 mg/ml sample buffer containing 5% sucrose, 2% SDS and bromophenol to the protein solution.

Electrophoretic separation of proteins was performed in the device for vertical gel electrophoresis Mini-PROTEAN Tetra Cell (Bio-Rad, USA). To identify proteins, the gel was stained in a solution of 0.125% Coomassie G-250 for 30 minutes. 3–9% solution of acetic acid was used to wash the gel in order to remove staining residues.

Turbidimetric analysis. The ability of fibrin to polymerize was tested by measuring the change in turbidity at 350 nm using POP spectrophotometer (Optizen, Daejeon, Korea). The experiments were carried out at 37 °C in 0.05 M Tris-HCl buffer, containing 0.13 M NaCl and 1 mM $CaCl_2$ at desAB fibrin final concentration of 0.1 mg/ml. To test the effect of inhibiting fibrin polymerization, the isolated D-dimer was added to the tested solution.

Calibration curves. Purified desAB fibrin and D-dimer were tested as calibrators for constructing curves for the above-described ELISA test systems developed by the O.V. Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine. After adding 100 µl of the conjugate into the wells with adsorbed monoclonal antibodies with the specificity to soluble fibrin and D-dimer in accordance, 10 µl of the obtained proteins in different concentrations were added. The content of the wells was mixed by gently shaking for 15–20 seconds. The wells were sealed with adhesive film and incubated at a temperature of 37 °C for 60 minutes. The microplate was washed with a washing solution 6 times. 100 µl of TMB(tetramethylbenzidine) substrate was added to the wells. The wells were sealed with an adhesive film and incubated at a temperature of 18–25 °C for 30 minutes in a dark place. 100 µl of the stop reagent was added to the wells in the same sequence as the TMB substrate. The optical density in the wells was measured in the two-wave mode (450 nm versus 620 nm). Based on the obtained data, calibration curves were constructed, on the Y axis of which the values of the optical density of the calibrators were marked, and on the X axis the corresponding values of the concentration of the calibrators in ng/ml.

Results and Discussion

Thrombophilia molecular markers, namely soluble fibrin (desAB fibrin) and D-dimer, were obtained from fibrinogen, which was isolated by salting out with Na_2SO_4 according to the above-described methodology. desAB fibrin was obtained by the enzymatic action of thrombin on the fibrinogen, while D-dimer was obtained due to fibrin stabilization and hydrolysis with the use of plasmin under the conditions described above. Purified proteins were tested for molecular weight and for the absence of impurities using the SDS-PAGE technique. As can be seen from the electrophoregram (Fig. 1), the obtained and purified proteins correspond to the appropriate molecular weight (190 kDa for D-dimer and 340 kDa for desAB fibrin) of the protein marker. The visualized protein bands indicate the high purity of the obtained proteins, since no additional bands are observed except for the target proteins.

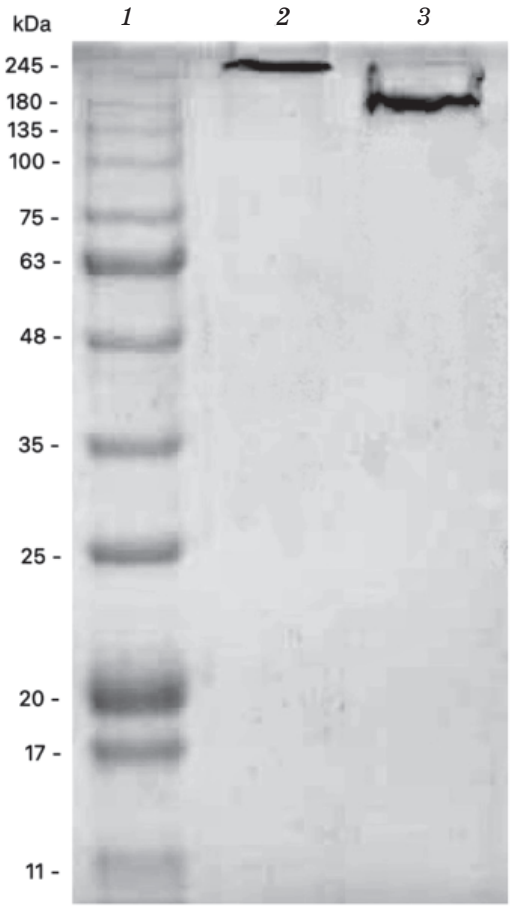

Fig. 1. **SDS-PAGE results using 7% polyacrylamide gel according to Laemmli:**
1 —Protein molecular weight markers;
2 — obtained desAB fibrin; *3* — obtained D-dimer

Turbidimetric measurements were performed to determine the ability of desAB fibrin to polymerize, as well as the ability of D-dimer to inhibit fibrin polymerization. This method of fibrin polymerization studying is based on the change in the turbidity during the formation of fibrin polymer. Turbidimetric studies were performed according to the methods described above, comparing the change in turbidity during the polymerization of native fibrin and under the conditions of the inhibitory action of the D-dimer. According to the obtained curves of turbidimetric measurements of fibrin polymerization (Fig. 2), the presence of D-dimer significantly reduced the rate and degree of polymerization, although it did not affect the lag-period of fibrin polymerization. Such results of turbidimetric studies are consistent with theoretical data on fibrin polymerization and its inhibition by D-dimer and confirm the functional activity of the obtained proteins [21].

Theoretically, the lag-period of polymerization reflects the stage of protofibril assembly from monomeric fibrin molecules. The rate of polymerization indicates the process of lateral association of protofibrils. Thus, the D-dimer is an ineffective inhibitor of protofibril assembly, while the D-fragment acts as an inhibitor precisely at this stage. According to the data of inhibitory properties studying of the D-dimer on the polymerization process of desAB fibrin, it is known that the mechanisms of interaction of D-dimer with fibrin monomer are different from the action of other specific inhibitors that contain D-domains. The reason is the conformational rearrangement of the D-domains, which occurs during the construction of the protofibril as a result of the interaction of the E_A–Da centers. Covalent cross-linking with the action of factor XIIIa fixes D-domains and their polymerization centers in the conformation that reproduces the conformation in the main assembly of the protofibril [22].

Obtained proteins were tested as calibrators to construct curves for the further use with immunoenzymatic test systems developed by the O.V. Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine. Such test systems are designed with the use of monoclonal antibodies and provide an opportunity to determine the concentration of soluble fibrin and D-dimer in blood plasma with high accuracy. Calibration curves for D-dimer (Fig. 3) and soluble fibrin (Fig. 4) were obtained by using the corresponding proteins. The principle of analysis of test systems is based on the method of solid-phase immunoenzymatic analysis in the sandwich modification using fibrin-specific and D-dimer-specific monoclonal antibodies and biotin-streptavidin amplification of a specific signal. The obtained curves indicate the possibility of using isolated proteins as calibrators for the corresponding immunoenzymatic test systems.

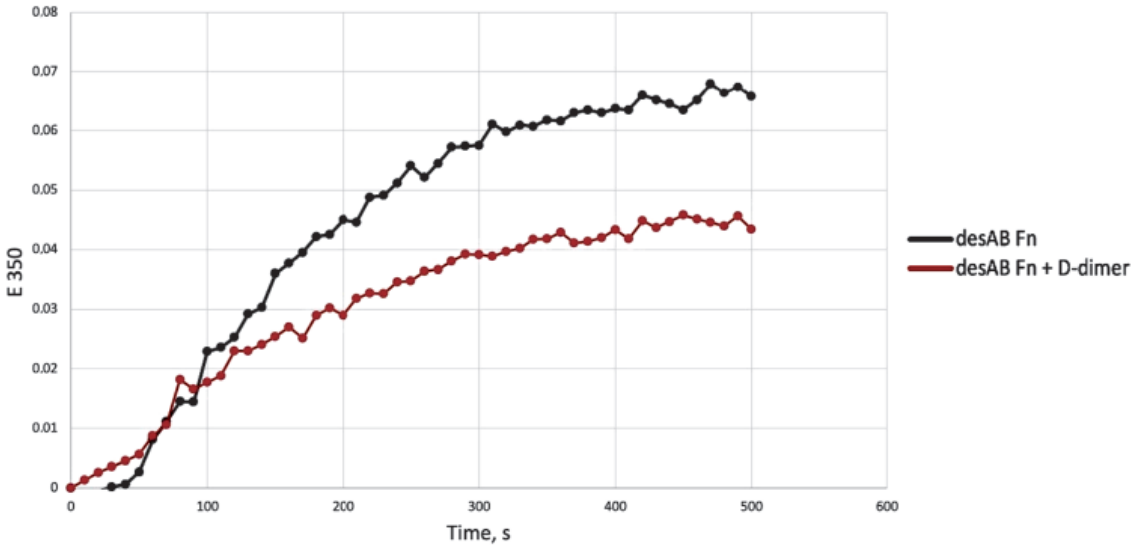

Fig. 2. **Turbidimetric measurement results of fibrin polymerization**

desAB fibrin polymerization (black graph) was performed by adding 0.25 U/ml of thrombin solution to the desAB fibrin solution at a final concentration of 0.1 mg/ml.

Inhibition of polymerization (red graph) was performed by adding an equimolar concentration of the obtained D-dimer and 0.25 units/ml of thrombin to the solution of desAB fibrin. The change in turbidity was measured spectrophotometrically at 350 nm.

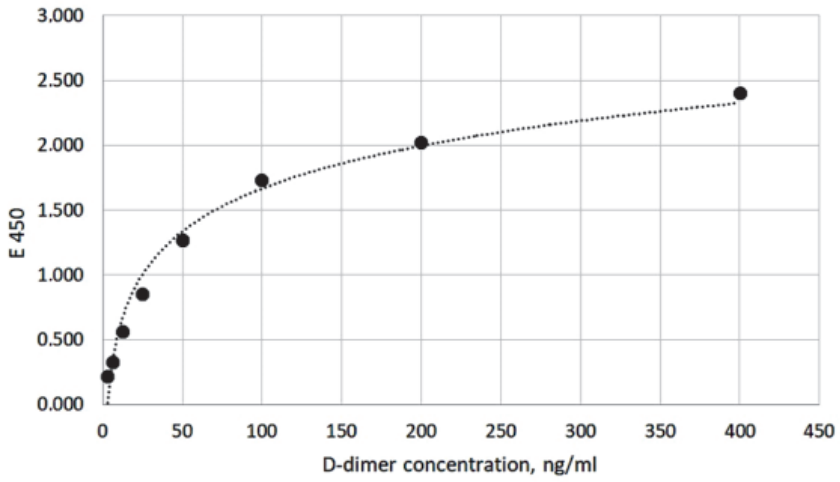

Fig. 3. **Calibration curve obtained by ELISA measurement of isolated D-dimer**

In the wells of the D-dimer determining ELISA microplate with adsorbed monoclonal antibodies 100 µl of the conjugate was added, as well as 10 µl of the obtained D-dimer in increasing concentrations.

After mixing and incubation at a temperature of +37 °C for 60 min, the microplate was washed for 6 times. 100 µl of TMB substrate was added to the microplate wells with further incubation at a temperature of +18–25 °C for 30 min. After adding 100 µl of the stop reagent, the optical density in the wells was measured in the two-wavelength mode at 450 nm versus 620 nm.

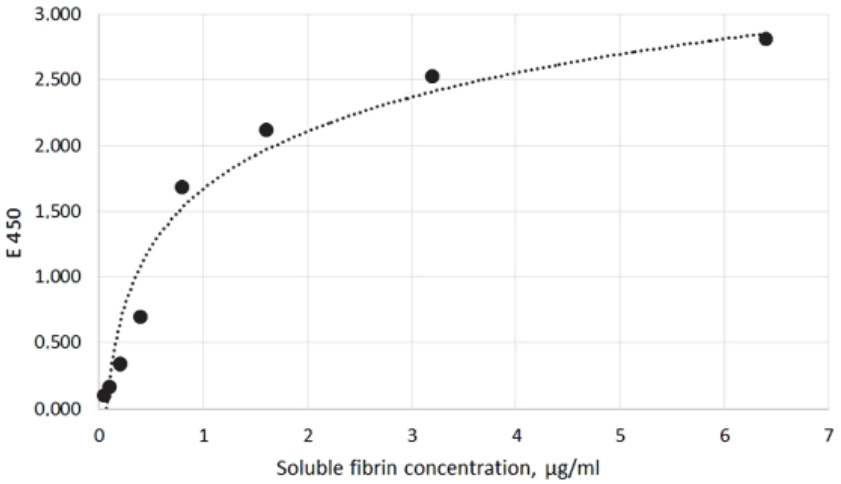

Fig. 4. **Calibration curve obtained by ELISA measurement of isolated desAB fibrin**

In the wells of the soluble fibrin determining ELISA microplate with adsorbed monoclonal antibodies 100 µl of the conjugate was added, as well as 10 µl of the obtained desAB fibrin in increasing concentrations.

After mixing and incubation at a temperature of +37 °C for 60 min, the microplate was washed for 6 times. 100 µl of TMB substrate was added to the microplate wells with further incubation at a temperature of +18–25 °C for 30 min. After adding 100 µl of the stop reagent, the optical density in the wells was measured in the two-wavelength mode at 450 nm versus 620 nm.

The developed by the O.V. Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine test systems for the determination of soluble fibrin and D-dimer have undergone successful clinical trials for the study of pathologies associated with thrombophilia. The developed and standardized methodology for obtaining protein calibrators for the above-described test systems can be used for the further production of test systems and their implementation in diagnostic practice.

Conclusion

The developed methodology of desAB fibrin and D-dimer isolation from human blood plasma by fibrinogen salting out with further fibrin polymerization by thrombin and hydrolysis with plasmin can be implemented for protein calibrators obtaining for their further use in immunodiagnostic test systems. Obtained proteins were tested for the quality control using electrophoresis in polyacrylamide gel for the absence of impurities and turbidimetric

measurements to detect polymerization ability of desAB fibrin and its inhibition by D-dimer, followed by the verification of proteins as calibrators by constructing the calibration curves for the quantitative concentration detection of thrombophilia markers by immunoassay.

Conflicts of interest

Authors declare no conflict of interest.

Authors Daria Korolova and Myroslav Syrko contributed to the work equally.

Funding

This article was prepared as a part of the scientific and technical work «Creation of recombinant therapeutic protein — activator of the blood clotting system» for the State Order under the contract DZ/104–2021 from March 9, 2021, of state registration 0120U104804 (scientific supervisor — Academician S. V. Komisarenko).

Research was supported by the ALLEA grant EFDS-FL2-02 provided to the Institution within the framework of Funding Line 2 of the"European Fund for Displaced Scientists" (EFDS).

Acknowledgments

The authors gratefully thank to Ukrainian Armed Forces for such an opportunity to live and work in a peaceful Kyiv.

REFERENCES

1. *Heit J.* Thrombophilia: common questions on laboratory assessment and management. *Hematology Am. Soc. Hematol. Educ. Program.* 2007, 1, 127–135. https://doi.org/10.1182/asheducation-2007.1.127
2. *Kwaan H., Lindholm P.* Fibrin and Fibrinolysis in Cancer. *Semin Thromb Hemost.* 2019, 45(4), 413–422. https://doi.org/10.1055/s-0039-1688495
3. *Takahashi D., Takahashi Y., Matsui M., Araki S., Kubo K., Sato H., Shirahata A.* Evaluation of hypercoagulability using soluble fibrin monomer complex in sick newborns. Pediatr. Int. 2013, 55(2), 151–156. https://doi.org/10.1111/ped.12050
4. *Chernyshenko V., Snezhkova E., Mazur M., Chernyshenko T., Platonova T., Sydorenko O., Lugovskoy E., Nikolaev V.* Blood coagulation parameters in rats with acute radiation syndrome receiving activated carbon as a preventive remedy. *Ukr. Biochem. J.* 2019, 91(2), 52–62. https://doi.org/10.15407/ubj91.02.052
5. *Lugovskoy E., Kolesnikova I., Platonova T., Lugovskaya N., Litvinova L., Kostyuchenko E., Chernyshenko T., Ganova L., Komissarenko S.* Simultaneous quantification of soluble fibrin and D-dimer in blood plasma for the assessment of the threat of thrombosis. *Clinical Medicine.* 2013, 11, 38-44.
6. *Komisarenko S., Deev V., Luhovskyi E., Kolesnikova I., Platonova T., Luhovska N., Kostyuchenko O., Chernyshenko V., Koroleva D., Chernyshenko T., Liksunov O., Kashirova O., Romanova E.* Application of immunoenzymatic methods for laboratory diagnosis of the threat of intravascular thrombus formation. Byhun V. Yu. 2019, 1-36.
7. *Stang L., Mitchell L.* Fibrinogen. *Methods Mol. Biol.* 2013, 992, 181–192. https://doi.org/10.1007/978-1-62703-339-8_14
8. *Schafer K., Goldschmidt E., Oostra D., Fish J., Russell T., Lurie F.* The clinical significance of ultra-high D-dimer levels. *J. Vasc. Surg. Venous Lymphat. Disord.* 2022, 10(1), 8–13. https://doi.org/10.1016/j.jvsv.2021.06.011
9. *Refaai M., Riley P., Mardovina T., Bell P.* The Clinical Significance of Fibrin Monomers. *Thromb Haemost.* 2018, 118(11), 1856–1866. https://doi.org/10.1055/s-0038-1673684
10. *Hoshino K., Muranishi K., Kawano Y., Hatomoto H., Yamasaki S., Nakamura Y., Ishikura H.* Soluble fibrin is a useful marker for predicting extracorporeal membrane oxygenation circuit exchange because of circuit clots. *J. Artif. Organs.* 2018, 21(2), 196–200. https://doi.org/10.1007/s10047-018-1021-x
11. *Horan J., Francis C.* Fibrin degradation products, fibrin monomer and soluble fibrin in disseminated intravascular coagulation. Semin Thromb Hemost. 2001, 27(6), 657–666. https://doi.org/10.1055/s-2001-18870
12. *Lugovskoy E, Kolesnikova I., Komisarenko S.* Usage of monoclonal antibodies for determination of localization of antigenic determinants and fibrin polymerization sites within fibrinogen and fibrin molecules and their application in test-systems for diagnostics and the threat of thrombus formation. *Biotechnologia Acta.* 2013, 6(4), 33–42. https://doi.org/10.15407/biotech6.04.033
13. *Litvinov R., Pieters M., Lange-Loots Z., Weisel J.* Fibrinogen and Fibrin. *Subcell Biochem.* 2021, 96, 471–501. https://doi.org/10.1007/978-3-030-58971-4_15
14. *Yao Y., Cao J., Wang Q., Shi Q., Liu K., Luo Z., Chen X., Chen S., Yu K., Huang Z., Hu B.* D-dimer as a biomarker for disease severity and mortality in COVID-19 patients: a case control study. *Journal of intensive care 2020*, 8, 49. https://doi.org/10.1186/s40560-020-00466-z

15. *Poudel A., Poudel Y., Adhikari A., Aryal B., Dangol D., Bajracharya T., Maharjan A., Gautam R.* D-dimer as a biomarker for assessment of COVID-19 prognosis: D-dimer levels on admission and its role in predicting disease outcome in hospitalized patients with COVID-19. *PloS one*, 2021, 16(8), e0256744. https://doi.org/10.1371/journal.pone.0256744
16. *Riley R., Gilbert A.* Widely Used Types and Clinical Applications of D-Dimer Assay. *Lab. Med.* 2016, 47(2), 90–102. https://doi.org/10.1093/labmed/lmw001
17. *Johnson E., Schell J., Rodgers G.* The D-dimer assay. Am J Hematol. 2019, 94(7), 833–839. https://doi.org/10.1002/ajh.25482
18. *Innocenti F., Lazzari C., Ricci F., Paolucci E., Agishev I., Pini R.* D-Dimer Tests in the Emergency Department: Current Insights. Open access emergency medicine 2021, 13, 465–479. https://doi.org/10.2147/OAEM.S238696
19. *Sikora-Skrabaka M., Skrabaka D., Ruggeri P., Caramori G., Skoczyński S., Barczyk A.* D-dimer value in the diagnosis of pulmonary embolism-may it exclude only?. Journal of thoracic disease 2019, 11(3), 664–672. https://doi.org/10.21037/jtd.2019.02.88
20. *Matsuo T., Kobayashi H., Kario K., Suzuki S.* Fibrin D-dimer in thrombogenic disorders. Semin Thromb Hemost. 2000, 26(1), 101–107. https://doi.org/10.1055/s-2000-9811
21. *Platonova T., Chernishenko T., Sokolovska L.* Characteristics of D-domain-containing specific inhibitors of polymerization on the fibrin self-assembly process. *Ukr. Biokhim. Zh.* 2006, 78(3), 124–129.
22. *Volkov G., Platonova T., Savchuk A., Gornytskaya O., Chernyshenko T., Krasnobryzhaya E.* Modern ideas about the system of hemostasis. Kyiv, Scientific opinion. 2005, 317.

СТАНДАРТИЗАЦІЯ МЕТОДОЛОГІЇ ОТРИМАННЯ БІЛКІВ КАЛІБРАТОРІВ ДЛЯ ІМУНОДІАГНОСТИЧНИХ ТЕСТ-СИСТЕМ ВИЗНАЧЕННЯ МАРКЕРІВ ТРОМБОФІЛІЇ

Дар'я Корольова[1], *Мирослав Сирко*[1, 2], *Євгеній Стогній*[1], *Надія Дружина*[1], *Тамара Чернишенко*[1], *Генрієтта Гоголинська*[1]

[1]Інститут біохімії ім. О.В.Палладіна НАНУ, Київ, Україна
[2]Київський національний університет імені Тараса Шевченка, Україна

E-mail: myroslaav@gmail.com

Найбільш точні лабораторні методи діагностики тромбофілії базуються на кількісному визначенні специфічних маркерів плазми крові, які виникають унаслідок активації коагуляційного каскаду. До основних таких маркерів належить розчинний фібрин та D-димер. Зміна концентрації цих маркерів може свідчити про зростання вмісту тромбіну та утворення розчинного олігомерного фібрину. Варто зазначити, що одночасне визначення маркерів тромбофілії дозволяє встановити кореляцію між накопиченням розчинного фібрину і фібринолізом та на сьогоднішні забезпечується лиш імуноферментним методом. Таким чином, використання імунодіагностичних тест-систем для визначення маркерів тромбофілії на сьогодні характризується високим рівнем актуальності. Важливими компонентами імунодіагностичних тест-систем є протеїни калібратори, стандартизація отримання яких відіграє ключову роль для точної побудови калібрувальних кривих та, як наслідок, отримання об'єктивних результатів.

Метою дослідження буле розроблення та стандартизація методології отримання розчинного фібрину та D-димеру для їхнього подальшого використання як протеїнів калібраторів в імунодіагностичних тест-системах для визначення маркерів тромбофілії.

Матеріали та методи. Розчинний фібрин та D-димер виділяли з плазми крові людини шляхом висолювання фібриногену з подальшою полімеризацією фібрину тромбіном та гідролізом із використанням плазміну. Контроль якості отриманих протеїнів проводили за допомогою SDS-PAGE та турбідиметричних вимірювань із подальшою перевіркою протеїнів як калібраторів для визначення маркерів тромбофілії імуноферментним методом.

Результати. Отримані протеїни відповідають необхідним вимогам та можуть використовуватись як калібратори для імунодіагностичних тест-систем. Розчинний фібрин та D-димер перевірені на відсутність домішок за допомогою SDS-PAGE. Турбідиметричні вимірювання показали здатність до полімеризації розчинного фібрину та інгібування полімеризації D-димером.

Висновок. Стандартизовану методологію виділення розчинного фібрину та D-димеру можна використовувати для отримання протеїнових калібраторів для відповідних імунодіагностичних тест-систем.

Ключові слова: протеїни калібратори; тромбофілія; розчинний фібрин; D-димер, імунодіагностичні тест-системи.