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DETECTION OF TERNARY COMPLEX OF FIBRIN DESAB WITH D-DIMER AND D-FRAGMENT OF FIBRIN

O. HRABOVSKYI[1], *M. SYRKO*[1, 2]

[1] Palladin Institute of biochemistry the National Academy of Sciences of Ukraine, Kyiv
[2] Educational and Scientific Center "Institute of Biology and Medicine"

E-mail: grabalexey@gmail.com

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Introduction. The fibrinogen molecule is a key protein in the haemostasis system, which is converted by thrombin into fibrin that polymerizes and forms a highly complex supramolecular structure [1]. This is done by stepwise cleavage of pairs of fibrinopeptides A (16 amino acid residues at the N-termini of the Aα chains) and fibrinopeptides B (14 amino acid residues at the N-termini of the Bβ chains) by thrombin, as a result, polymerization sites "A" and "B" are exposed on the fibrin molecule, while the complementary sites "a" and "b" that are located in the γ- and β-nodules are preexisted in the fibrinogen molecule [2].

The interaction of these sites, primarily "A:a", leads to the formation of a protofibril, which can be considered the first stage of fibrin polymerization. The second stage which is the lateral association of protofibrils is driven by the interaction of "B:b" polymerization sites, αC-regions of fibrin molecules and the interaction of D-regions of fibrin molecules that belongs to the neighbouring protofibrils [1, 3, 4].

Model systems are one of the approaches to study the mechanism of fibrin self-assembly. Experimental data obtained on such models can be used to elucidate the mechanisms of interaction of individual functional domains of fibrin molecules and characterize the polymerization process in general. Therefore, the aim of this work is to study the intermolecular interactions of fibrin with D-domain-containing fragments of fibrin(ogen): D-dimer and D-fragment.

Methods. Human fibrinogen was obtained from the human blood plasma by salt extraction using 16 % Na_2SO_4. The content of protein coagulated by thrombin — 96–98%. The precipitated fibrinogen was diluted in 0.15 M NaCl, the solution was frozen and stored with −20 °C [5]. Fibrin desAB was obtained by thrombin action on fibrinogen with following dissolving in 0.125 % acetic acid. D-dimer and D-fragment of fibrin were obtained by plasmin hydrolysis of cross-linked fibrin desAB or fibrinogen respectively with following purification using size-exclusion chromatography on Sephacryl S-300 HR and characterized by SDS-PAGE.

Analytical size-exclusion chromatography for the detection of molecular complexes was performed on the Sepharose 6B column (30×0.5 cm). Components of the analyzed mixture (0.8 ml) were separated by standard chromatography protocol: speed of elution — 0.5 ml/min; collected samples volume —0.5 ml. Optical density of collected samples was measured by spectrophotometer POP (Optizen, Daejeon, Korea). Composition of each sample was analyzed by SDS-PAGE. Relative amounts of studied compounds in samples were analyzed using densitometry of scanned electropherograms with Totallab TL100 software [6].

Molecular modeling of complexes formed by fibrin desAB and its fragments were performed using UCSF Chimera 1.16 on the basis on earlier developed protofibril structure [7]. This structure was prepared for molecular docking by removing and truncating all chains except those belonging to the sole molecule of fibrin and corresponding D-dimer, adding hydrogens with subsequent energy minimization of the complex. The structure of the D-region (PDB ID:1LTJ) was prepared in the same way. The protein-protein molecular docking was performed using HDOCK web server [8].

Results. To investigate the complex formation between fibrin desAB (fibrin with both cleaved fibrinopeptides A and B) and its degradation products, we used size-exclusion chromatography,

which allows separating different fractions of proteins depending on their molecular weight. It also can be an option for evaluation if larger, high-affinity protein-protein complex is formed [9].

To verify and calibrate the retention volume of the mixture components: fibrin monomer and fibrin(ogen) fragments, each component was applied to the column separately. The fibrin exit zone corresponded to a volume of 7 ml, D-dimer to 8 ml, and D-fragment to 9.5 ml.

To obtain the fibrin-DD-D complex we have composed a mixture of fibrin desAB (1 mg/ml) with D-dimer and D fragment in the molar ratio 0.8:1:1. Fragments were added to the 0.05 M Tris-HCl buffer pH 7.4 with 0.4 M NaCl and 0.001 M $CaCl_2$. Monomeric fibrin desAB dissolved in 0.125 % acetic acid was added to the solution of fragments in a small volume. The mixture was applied to the Sepharose 6B column after 30 min of incubation at ambient temperature. The elution profiles were monitored and the composition of obtained fractions were analyzed by SDS-PAGE. The formation of a stable ternary complex «desAB-DD-D» was observed using SDS-PAGE (Fig. 1).

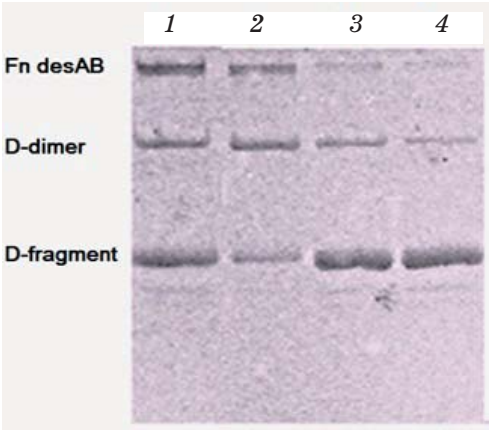

Fig. 1. **SDS-PAGE of fractions eluted from the Sepharose 6B column:**
1 — mixture of purified fibrin desAB, D-dimer and D-fragment that was applied to the column and was used as a marker; *2* — fraction eluted at the volume 5.5 ml; *3* — fraction eluted at the volume 8 ml; *4* — fraction eluted at the volume 9.5 ml

In particular, the appearance of D- and DD-fragments in the elution zone of 5.5 ml, which does not overlap with the elution zone of individual fragments (7.5–9.5 ml), was detected, indicating the formation of a ternary complex. Densitometry of electropherograms using TotalLab TL-100 demonstrated that the average densities of pixels in bands of fibrin desAB, D-dimer and D-fragment were equal. It means that the ternary complex of fibrin desAB with D-dimer and D-fragment was composed in the approximate ratio of fibrin desAB, D-dimer and D-fragment 1:1:1.

Molecular docking in the HDOCK software was used to establish the spatial arrangement of the D-fragment in relation to the fibrin desAB molecule bound to the D-dimer. The theoretical binding pose is shown in Fig. 2.

Discussion. For the first time, we have obtained the molecular complex of fibrin desAB, D-dimer and D-fragment using size-exclusion chromatography.

Since protofibrils are formed due to intermolecular interactions of fibrin molecules: the central E-region of one molecule interacts with the peripheral D-regions of the other two (DD-E triad), the formation of the DD-E triad occurs primarily due to the interaction of the “A:a” polymerization sites. Therefore, the complex formation of fibrin desAB with D-dimer is understandable and occurs via the mentioned sites. However, the involvement of another D-region in the complex is not obvious.

The main question raised is what sites of interaction are obedient for the involvement of the D-dimer and D-fragment in fibrin desAB. There are several scenarios for how this interaction occurs. The first possibility is that fibrin desAB binds to the D-dimer through the interaction of one

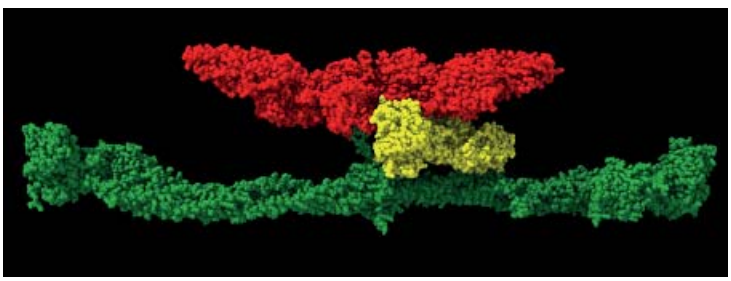

Fig. 2. **Molecular complex of fibrin desAB with D-dimer and D-fragment:**
Green color refers to fibrin molecule, red color — D-dimer and yellow color -— D-fragment

pair of "A:a" sites, while the second pair is involved in the binding of the D-region. Another variant involves the interaction of two pairs of "A:a" sites belonging to fibrin desAB and D-dimer, and the D-fragment is involved through the interaction of the "B:b" site. However, the fact that only one D-fragment is involved remains unclear in this case, since there are two pairs of "B:b" sites. It is possible that the attachment of the second D-fragment is impossible due to conformational clashes caused by conformational changes in the ternary complex.

Conclusions. We obtained and characterized the ternary complex of fibrin desAB, D-dimer and D-fragment by size-exclusion chromatography followed by SDS-PAGE. Further study of the structure and properties of this complex may clarify certain issues related to fibrin polymerization, namely the process of protofibril formation and their spatial branching.

Keywords: fibrin; D-dimer; D-region; fibrin polymerization; docking.

Authors' contribution. OH worked on molecular modeling and size-exclusion chromatography, MS worked on size-exclusion chromatography and SDS-PAGE.

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