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ROLE OF FIBRINOGEN AND FIBRIN IN INITIATION OF THROMBIN GENERATION

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Background. *Thrombin is the key enzyme of blood coagulation that converts fibrinogen into fibrin and activates multiple coagulation factors. However, the temporal relationship between thrombin-mediated fibrin formation and the availability of thrombin for other physiological substrates during the initiation of coagulation remains insufficiently understood.* ***Objective.*** *To investigate the role of fibrinogen and fibrin in regulating thrombin activity during the initiation phase of thrombin generation in plasma.* ***Methods.*** *Thrombin generation was initiated in human plasma using activated partial thromboplastin time (APTT) reagent or Thromborel. Fibrin formation was monitored turbidimetrically at 405 nm. Thrombin amidolytic activity toward the chromogenic substrate S2238 was measured spectrophotometrically at 405 nm. Fibrin polymerization was inhibited with the synthetic peptide GPRP. The effects of plasma dilution and inhibition of fibrin polymerization on the temporal profiles of fibrin formation and thrombin activity were analyzed.* ***Results.*** *During initiation of thrombin generation with both APTT-reagent and Thromborel, the beginning of cleavage of S2238 by thrombin coincided with the end of fibrin clot formation, marked by turbidimetric curve. At 1:3 plasma dilution, fibrin formation lag time was 210 s, amidase activity lag phase was 410 s. At 1:10 dilution, the lag time for fibrin formation was 73 s, amidase activity was 200 s, indicating that dilution (and lower fibrinogen) accelerates thrombin generation. With 1 mM GPRP, fibrin and amidase activity lag times increased by an order of magnitude.* ***Conclusions.*** *Thrombin generated during initiation of thrombin generation acts primarily on fibrinogen, remaining bound to the molecule throughout its conversion into fibrin and clot formation. Its amidase activity emerges only after protofibrils undergo lateral association into fibrin clot. Once the binding sites in the fibrin clot are saturated, thrombin becomes available for other substrates (FV, FVIII, FXI) and physiological functions.*

K e y w o r d s: thrombin, fibrinogen, fibrin, thrombin generation assay.

Thrombin is a multifunctional enzyme [1]. As the main enzyme of the hemostasis system, it ensures blood coagulation and the formation of clots to maintain the integrity of the cardiovascular system [2-4]. After clot formation, thrombin activates the anti-coagulant mechanisms of the hemostasis system, inactivating the tenase and prothrombinase complexes [5, 6]. Thrombin's multifunctionality is realized over time and through the compartmentalization of its action in different parts of the cardiovascular system with the participation of LDL and PAR receptor proteins, thrombomodulin, and its interaction with the glycocalyx and vascular endothelium of the circulatory system [7-9]. Therefore, the determination of thrombin activity during its *in vitro* generation is an important diagnostic method for assessing the activity of the components of the coagulation and anti-coagulation systems of hemostasis, their balance, and for determining the state of the hemostasis system [10, 11].

The activation of hemostasis coagulation system has three phases, namely: the initiation phase of coagulation, the amplification phase of thrombin activity, where the maximum thrombin activity is determined, and the resolution phase, which is characterized by endogenous thrombin potential of blood plasma [10, 11]. The most complex and important for thrombin generation and fibrin clot formation is the initiation phase, during which the activity of the formed thrombin is focused only on the transformation of fibrinogen into fibrin, the formation of a fibrin clot, and does not act on other enzyme substrates [12].

The amplification phase of thrombin activity begins after completion of initiation phase with the formation of fibrillar structure of the clot [13, 14]. It is associated with completion of the fibrinogen into fibrin transformation and thrombin binding sites saturation on the polymeric fibrin. It is assumed that the excess concentration of thrombin, which is formed within the fibrin clot both *in vitro* and *in vivo* in case of major bleeding, is necessary for the rapid completion of clot and its surface formation [15]. This is primarily due to the activation of FXIII to FXIIIa, followed by the cross-linking of fibrin via γ-γ chains, the inclusion of α2-AP, PAI-1 in the clot, the activation of platelets, and the cross-linking of IIbIIIa with polymeric fibrin to form a strong and protease-resistant thrombus surface [16, 17].

Due to the absence of amidase activity in the initiation phase of coagulation system activation, less attention has been paid to the relationship between fibrin clot formation and thrombin formation than to subsequent phases of thrombin generation [10-18]. This work presents the results of a study on the temporal relationship between the stages of clot structure formation and thrombin generation in human blood plasma in vitro.

Material and Methods

Activated partial thromboplastin time (APTT) reagent from Granum (Ukraine) and recombinant tissue plasminogen activator (t-PA) from Boehringer Ingelheim (Germany). S2238 pNA-labeled peptide substrate was purchased from Chromogenix, Gly-Pro-Arg-Pro (GPRP) pNA-labeled peptide substrate - from Sigma-Aldrich, St. Louis, USA). Thrombin-like enzyme Ancistron was purified from the venom of A. halys halys using Blue-Sepharose (Sigma-Aldrich, St. Louis, USA).

Donor blood samples were collected in 3.8% sodium citrate (1 part sodium citrate and 9 parts blood, pH 7.4). Plasma was separated from blood cells within one hour after blood collection by centrifuging the latter at 1500 g for 30 min. Aliquots of plasma were stored at -20°C.

Blood plasma coagulation was studied using the turbidimetric method by recording the turbidity of the fibrin clot at 405 nm on an SF-2000 Spectrophotometer [19]. The clot was formed in plastic cuvettes to which 0.02 M HEPES buffer containing 0.15 M NaCl, 0.005% Tween-20, pH 7.4, 40 µl or 133 µl of blood plasma and 50 µl of the APTT reagent or 2,0 µl TF were added. Chromogenic substrate S2238 at 0.3 mM concentration was used to determine thrombin amidase activitie. The plasma coagulation process was initiated by adding 25 mM $CaCl_2$. The final volume of the reaction mixture was 400 µl.

Non-clotting plasma was obtained by adding proteolytic enzyme from the venom of *Agkistrodon halys halys.* After 30 min of incubation of plasma with this enzyme (0.02 mg/ml) at 37°C, the Aα chains were hydrolyzed while the Bβ and γ chains remained intact but plasma lost clotting ability [20].

The results were processed using the standard Excel statistical program. The average values of the parameters and their standard deviation were determined.

Results and Discussion

Activation of the human plasma coagulation system begins with the initiation phase, which proceeds through two activation pathways – the primary extrinsic and the additional intrinsic [5, 6]. *In vitro*, the initiator of the extrinsic pathway is tissue factor (TF), whereas the initiator of the intrinsic pathway is the APTT reagent.

A study of the temporal relationship between clot formation and thrombin generation at the initiation stage of coagulation activation showed, as seen in Fig. 1, that in both systems at the initiation stage of coagulation activation, the start of recording thrombin generation by its amidase activity coincides in time with the completion of clot formation in blood plasma. Thrombin generation accelerates sharply in both systems after the completion of the clot structure formation.

In the system with an APTT reagent, a high level of spontaneous activity is observed, which probably belongs to three enzymes capable of being activated by the APTT reagent, namely: FXII, kallikrein, and FXI, which, after activation, hydrolyze the thrombin substrate [21]. The kinetics of S2238 cleavage indicates a simultaneous and complete activation of all non-specific enzymes by the APTT reagent, Fig. 1 (right panel).

The obtained result indicates the existence of a temporal sequence between the appearance of fibrin clot and the amidase activity of generated thrombin, namely: thrombin generated during the initiation phase of coagulation begins to exhibit its amidase activity at the stage of clot completion in plasma. This may reflect a temporal relationship between thrombin generation and the stages of clot formation

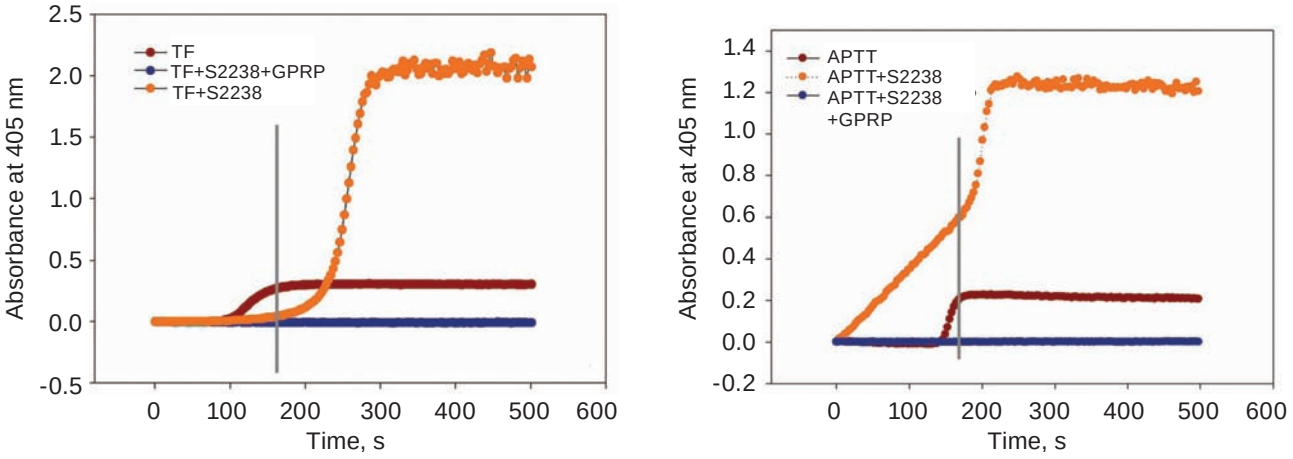

Fig. 1. Comparative analysis of plasma clotting and amidase activity growth with activation by Thromborel (tissue factor, left) or APTT reagent (right). Each curve represents the average of two experiments: turbidimetric clotting without S2238 (dark red, 405 nm); thrombin generation with S2238 (orange, 405 nm); both in the presence of 1 mM GPRP peptide (dark blue). Vertical segments indicate that amidase activity acceleration coincides with completion of fibrillar clot formation

in human plasma, initiated by TF or APTT reagent, and may suggest the involvement of fibrinogen (Fg) and fibrin in the regulation of prothrombin activation during initiation.

Fg is at the boundary between the system of prothrombin activation into thrombin, which is generated by action of TF-FVIIa-FXa and FIXa-FXa complexes, and formation of fibrin clot, Fig. 2. The concentration of Fg in plasma is normally in range of 5.8 – 11.6 μM. At the beginning of the coagulation initiation phase, Fg is the only substrate for thrombin, under the action of which it transforms into desA-fibrin, and the latter begins to form polymeric clot structures, Fig. 2. S2238 cannot affect these processes due to its weak affinity for thrombin and its weak influence on fibrin polymerization [1-5].

It should be noted that the cleavage of fibrinopeptides A and the formation of desA-fibrin occurs at sub- and nM concentrations of thrombin [11]. The formation of desA-fibrin protofibrils and their lateral association stimulates the cleavage of fibrinopeptides B and the formation of desAB-fibrin fibrils and the clot as a whole. The amidase activity of thrombin begins to manifest itself only after the formation of a fibrin clot, as is clearly seen in Fig. 3 (plasma dilution 1 : 10). During the time of lateral association of protofibrils and due to the high affinity of thrombin for the fibrin that is forming and formed into a clot, the generated thrombin saturates the binding sites on the fibrin fibrils of the clot, transforming it into a thrombin depot and begins to be released into the interfibrillar space of the clot, Fig. 2 [10].

Thrombin, which in increasing concentration enters into interfibrillar space of the blood plasma clot, switches to the activation of FVIII, FV and FXI, which form tenase (FVIIIa - FIXa- FXa-PL) and prothrombinase (FVa-FXa-PL) complexes, causing a “burst” in the growth of the amidase activity of thrombin. It is likely that in this way Fg and fibrin sequentially and in different forms regulate the initiation of activation of the coagulation system.

It should also be noted that under conditions of weak prothrombin activation, Fg can block the polymerization of desA-fibrin at the soluble fibrin stage [22]. This delay the formation of protofibrils and the fibrin clot, which serves as thrombin reservoir required for the further activation of the initiation phase of plasma coagulation.

To determine the role of polymeric fibrin structure in the initiation of thrombin generation, we used the highly specific polymeric fibrin formation inhibitor – GPRP peptide [23]. As the data presented in Fig. 1 show, 1 mM GPRP completely suppressed not only the clot formation in blood plasma initiated by TF or aPTT reagent, but also completely inhibited the initiation stage of thrombin’s amidase activity generation in both systems.

In contrast to donor plasma, in nonclotting plasma the (TF–FVIIa)–FXa complex exhibited had no lag phase and activated thrombin generation al-

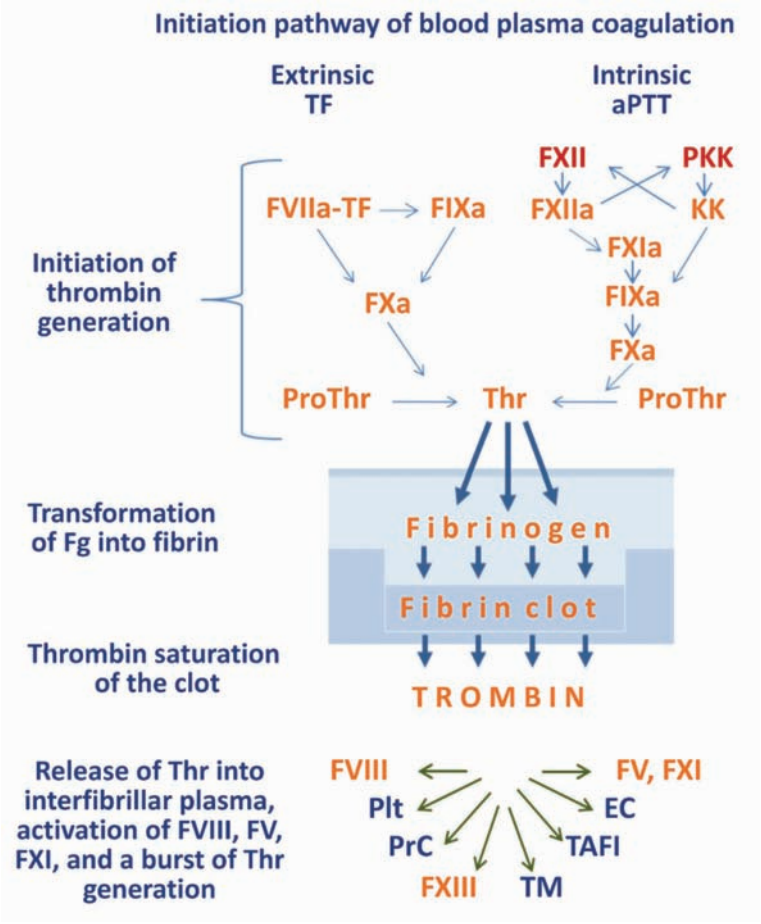

Fig. 2. Schematic representation of thrombin generation initiation and subsequent participation of thrombin in the reactions of initiation phase of plasma coagulation. The figure illustrates the sequence of reactions of thrombin initiation, starting with the formation of TF-FVIIa-FXa and FIXa-FXa complexes, clot formation, and participation of its structures in the initiation of thrombin generation. Plt – platelets, EC – endothelial cells, PrC – protein C, TAFI – thrombin activatable fibrinolysis inhibitor, FXIII – transglutaminase, TM – thrombomodulin

most linearly and significantly more slowly. Thus, Fig. 3 shows the thrombin generation curve initiated by TF (Thromborel, Siemens, Germany). In nonclotting plasma, the release of the p-NA label from the S2238 thrombin substrate in 500 s was ~ 0.4 a.u. at 405 nm. At the same time, in donor plasma in the presence of TF or aPTT reagent, the entire substrate is destroyed in 250 and 230 s, respectively, Fig. 3. This result indicates that TF-FVIIa-FXa and FIXa-FXa complexes in blood plasma are weak activators of thrombin generation in the absence of polymeric fibrin (clot).

On the other hand, this study indicates the absolute necessity of Fg and fibrin participation in the formation of the fibrin clot to ensure rapid and effective thrombin generation in donor plasma, Fig. 1.

The effect absence of 1 mM GPRP peptide on thrombin generation in defibrinated blood plasma is direct evidence of the necessity of fibrin in the form of a clot for the effective initiation of thrombin generation. The mechanism of fibrin clot's participation in stimulating prothrombin activation is not fully understood.

To clarify the effect of blood plasma dilution on clot formation and the initiation of thrombin generation (Fig. 4), we compared the onset of thrombin generation, as measured by its amidase activity, in a system containing plasma + S2238 + Thromborel (TF). It was 410 s with a 1:3 plasma dilution, while the lag period before the start of clot formation was 210 s.

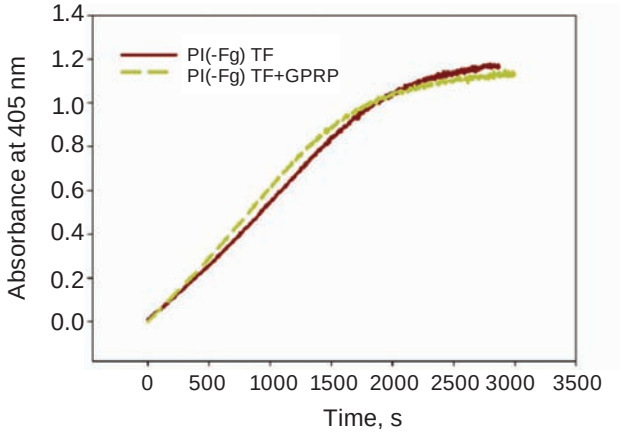

Fig. 3. The effect of 1 mM GPRP peptide on thrombin generation initiated by tissue factor in nonclotting human blood plasma

In the 1:10 diluted plasma, lag period for clot was 73 s, and for amidase activity, it was 200 s. The decrease in fibrinogen concentration in the 1:10 diluted plasma accelerated thrombin generation. These data confirm our hypothesis that fibrinogen acts not only as a substrate for thrombin but also as an inhibitor of fibrin polymerization (Antithrombin I), since it increases the lag period for clot formation by three times. Evidently, inhibition of fibrin polymerization by fibrinogen is reduced much more strongly when fibrinogen concentration is diluted tenfold than threefold. Secondly, the capacity of fibrin clot as a thrombin reservoir, under threefold plasma dilution, significantly exceeds the concentration of generated thrombin. This increases the time required to saturate the clot with thrombin and prolongs the tenase and prothrombinase complexes formation twofold, thereby delaying the burst of amidase activity in plasma.

Conclusion. Thrombin generation, as measured by amidase activity, appears and begins to accelerate simultaneously with the completion of fibrin clot's fibrillar structure formation. Clearly, the three-dimensional structure of polymer fibrin is essential for thrombin accumulation [24]. The clot serves as a thrombin reservoir that enables the realization of multifunctional potential of thrombin (Fig. 2). As mentioned earlier, after clot saturating with thrombin, excess of the latter switches to activating FVIII, FV, and FXI factors, leading to the formation of tenase and prothrombinase complexes. These complexes cause a burst in thrombin activity, which is necessary for the final stages of clot and thrombus formation. *In vitro,* this allows for the determination of important diagnostic parameters of thrombin generation, such as the lag period of thrombin generation initiation, the maximum rate of prothrombin activation, maximum thrombin activity and concentration, and the endogenous thrombin potential of the patient's blood plasma. *In vivo,* a high concentration of thrombin is essential for platelet activation, the completion of clot formation, and the formation of a thrombus surface structure resistant to the action of proteolytic enzymes and the fibrinolysis system.

Thus, thrombin generation during the initiation phase of coagulation activation is a process strictly directed at fibrin clot formation. The observed pattern of thrombin activity generation, linked to fibrin

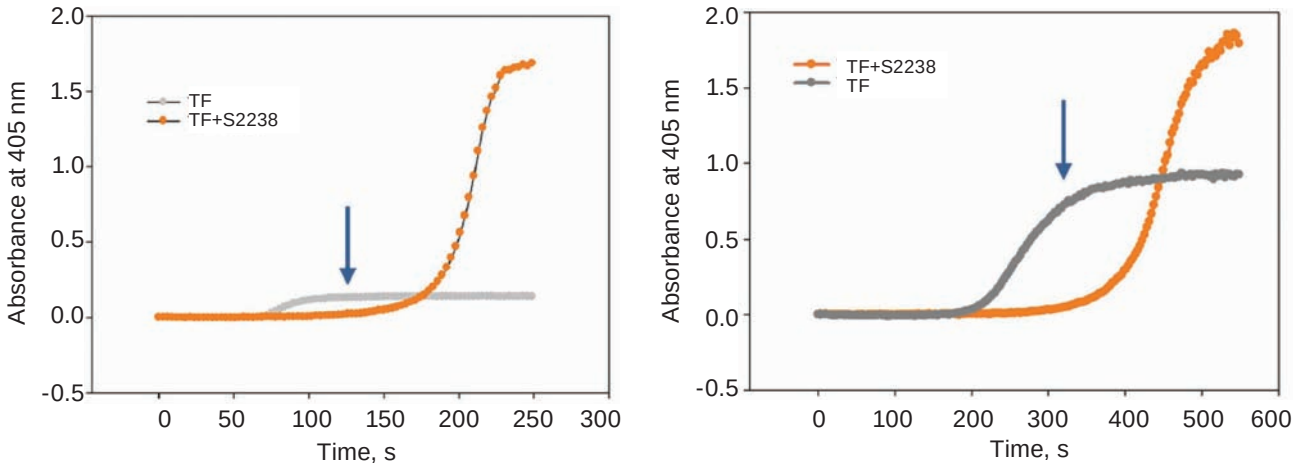

Fig. 4. The effect of 1:10 and 1:3 blood plasma dilution on the parameters of coagulation activation and the initiation of thrombin generation in blood plasma, where coagulation is initiated by tissue factor (Thromborel)

clot formation, can indicate the balance between the activation of the coagulation system and the anticoagulation hemostasis of blood plasma.

Author contributions. A. V. Udovenko – conducted the experiments and edited the manuscript; Ye. M. Makogonenko – conceived the study, supervised the research, and wrote the manuscript; V. O. Chernyshenko – supervised the research and edited the manuscript.

Conflict of interest. The authors have completed the Unified Conflicts of Interest form at http:// ukrbiochemjournal.org/wp-content/uploads/2018/12/ coi_disclosure.pdf and declare no conflict of interest.

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РОЛЬ ФІБРИНОГЕНУ ТА ФІБРИНУ В ІНІЦІАЦІЇ УТВОРЕННЯ ТРОМБІНУ

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Вступ. Тромбін є ключовим ензимом системи зсідання крові, який перетворює фібриноген на фібрин і активує численні фактори згортання. Однак часовий взаємозв'язок між тромбін-опосередкованим утворенням фібрину та доступністю тромбіну для інших фізіологічних субстратів на стадії ініціації згортання залишається недостатньо вивченим. **Мета.** Дослідити роль фібриногену та фібрину в регуляції активності тромбіну на стадії ініціації генерації тромбіну в плазмі. **Методи.** Генерацію тромбіну в плазмі крові людини ініціювали за допомогою реагенту активованого часткового тромбопластинового часу (АЧТЧ) або Thromborel. Формування фібрину контролювали турбідиметрично при 405 нм. Амідолітичну активність тромбіну щодо хромогенного субстрату S2238 визначали спектрофотометрично при 405 нм. Полімеризацію фібрину інгібували синтетичним пептидом GPRP. Аналізували вплив розведення плазми та інгібування полімеризації фібрину на часові профілі формування фібрину й активності тромбіну. **Результати.** Під час ініціації генерації тромбіну як реагентом АЧТЧ, так і Thromborel, початок розщеплення S2238 тромбіном збігався із завершенням формування фібринового згустку, що визначали за турбідиметричною кривою. У разі розведення плазми 1:3 час затримки формування фібрину становив 210 с, а лаг-фаза амідолітичної активності – 410 с. За розведення 1:10 час затримки формування фібрину становив 73 с, а лаг-фаза амідолітичної активності – 200 с, що свідчить про прискорення генерації тромбіну при розведенні плазми (і зниженні концентрації фібриногену). За наявності 1 мМ GPRP лаг-фази формування фібрину та амідолітичної активності збільшувалися приблизно на порядок. **Висновки.** Тромбін, що утворюється на стадії ініціації генерації тромбіну, насамперед взаємодіє з фібриногеном і залишається зв'язаним із цією молекулою протягом усього процесу її перетворення на фібрин і формування згустку. Його амідазна активність проявляється лише після латеральної асоціації протофібрил у фібриновий згусток. Після насичення сайтів зв'язування у фібриновому згустку тромбін стає доступним для взаємодії з іншими субстратами (FV, FVIII, FXI) та виконання інших фізіологічних функцій.

Ключові слова: тромбін, фібриноген, фібрин, аналіз генерації тромбіну (TGA).

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