UDC 612.115:612.128 https://doi.org/10.15407/biotech16.02.050

DETERMINATION OF THROMBIN AND PLASMIN ACTIVITY IN HUMAN BLOOD PLASMA USING THE TURBIDIMETRIC CURVE OF CLOT FORMATION AND DISSOLUTION

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Received 2023/03/09
Revised 2023/04/03
Accepted 2023/04/28

Thrombin and plasmin are the terminal enzymes of the activation pathway of the coagulation and fibrinolysis systems of human blood plasma, respectively. Their concentrations indicate the degree of functional readiness of each of the systems to respond to disturbances in the hemostasis system, and the ratio of the rates of formation and dissolution of a plasma clot *in vitro* indicates the degree of balance between the coagulation and fibrinolysis systems (1). Thrombin is a multifunctional enzyme, which prompted the development of numerical methods for determination its concentration in blood plasma, using predominantly the enzyme amidase activity to peptide substrates labeled by p-nitroaniline (pNA) or fluorescent mark (*1, 2*). However, it was shown that the curve of amidase activity for thrombin does not coincides with such of clot turbidity. Therefore, the method of determining the rate of activation and concentration of thrombin based on its amidase activity does not reflect the rate of activation of thrombin in the initial period of blood plasma coagulation, in which protofibrils are formed and thrombin activity is directed only to the formation of fibrin. The thrombin, formed by the coagulation system in the lag period, exhibits maximum activity in relation to the fibrinogen substrate, while the rate of protofibril formation depends on the thrombin concentration.

Aim. The purpose of the work was to develop a method for determination the activity of thrombin, which is based on the turbidimetry curve (TDC) of the formation and dissolution of a blood plasma clot. At the same time, the theoretical analysis of the dissolution phase of the blood plasma clot by the fibrinolytic system allowed to propose a method for determination the concentration of the formed plasmin using its amidase activity.

Methods. Donor blood samples were collected in 3.8% sodium citrate (1 part of sodium citrate and 9 parts of blood, pH 7.4). Plasma was separated from blood cells within 1 hour after blood collection by centrifugation the latter at 1200 g for 20 min. Aliquots of plasma were stored at −20 °C.

Blood plasma coagulation was studied by the turbidimetric method by recording the turbidity of the fibrin clot at 405 nm on a SF 2000 spectrophotometer.

The clots were formed in plastic cuvettes, into which 0.05 M HEPES buffer containing 0.15 M NaCl, 0.005% Tween-20, pH 7.4, 40 µl of blood plasma, t-PA to a final concentration of 75 IU/ml and 50 µl of APTT reagent were added. The plasma coagulation process was initiated by adding 25 mM $CaCl_2$. The final volume of the reaction mixture was 400 µl. Processing of the results was performed a standard statistical program in *Excel*. The mean values of the parameters and their standard deviation were determined.

Results. To determine the concentrations of thrombin and plasmin, TDCs of the formation and dissolution of blood plasma clots, initiated by the APTT reagent, were used. A typical experiment in which TDC was used to determine τ values at thrombin concentrations from 0.1 to 0.5 NIH per mlb is shown in the Fig. Based on τ values obtained in 3 experiments, a calibration curve was constructed in the coordinates $1/\tau$ — [Thr] (the rate of protofibrils formation in s^{-1} vs thrombin concentration in NIH units in 1 ml). The activity of thrombin — 1 NIH in 1 ml — corresponded to a concentration of 27,7 nM.

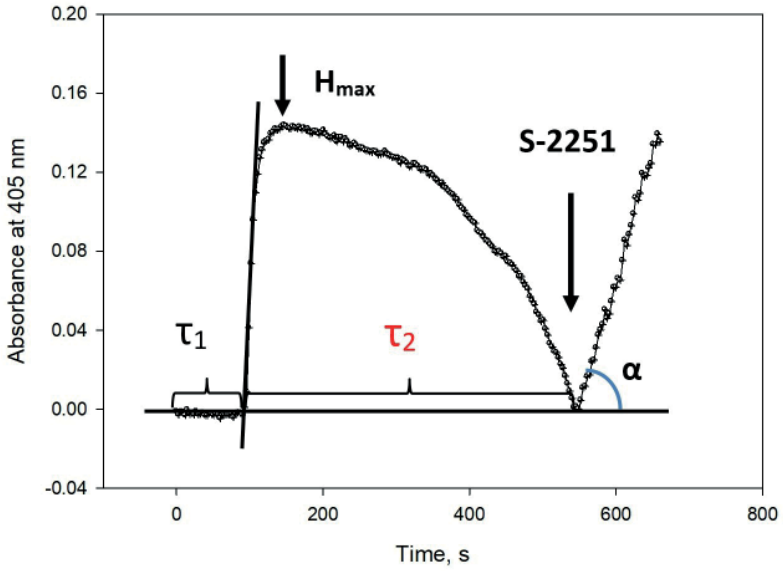

Figure. **Determination of thrombin and plasmin activity, which are formed during the formation and dissolution of a fibrin clot in blood plasma in the presence of t-PA and the peptide substrate S 2251 (H-D-Val--Lys-pNA), added at the moment of complete dissolution of the clot, using parameter τ and amidase activity of plasmin (V), respectively:**

τ_1 — lag period of clot formation; τ_2 — time of clot existence; H_{max} — maximum clot turbidity, point of transition from clot formation stage to clot dissolution stage; S-2251 — moment of introduction of plasmin substrate into the dissolved clot; α — angle of inclination of S-2251 cleavage curve to the abscissa axis; tgα is equal to the amidase activity of the formed plasmin (V)

In 6 independent experiments based on TDC, the following values of τ and V for thrombin and plasmin were found, respectively, 0.095±8.83 s^{-1} and 0.062±0.12 optical units (o.u.)/min, which corresponded to their concentrations of 2.77 nM for thrombin and 22.3 nM for plasmin. Considering that the concentration of prothrombin and Glu-Pg in the reaction medium was 100 and 140 nM, respectively, the% concentrations of activated thrombin and plasmin to such of proenzymes were 2.9% and 15.9%, respectively. The thrombin concentration ratio to such of plasmin was ~ 1:8. It turned out to be sufficient activation 2.9% concentration of prothrombin for the protofibrils formation, and 15.9% concentration of Glu-Pg for complete dissolution of the clot.

Discussion. Activity and concentration of thrombin were determined by its ability to transform the natural substrate fibrinogen into fibrin and form the basic structure of the clot — protofibrils. Determination of plasmin activity was based on direct measurement of plasmin amidase activity at the moment when the three-dimensional structure of the plasma clot was completely destroyed and the optical density in the cuvette reached zero. Calculation of plasmin activity was carried out based on data of the plasmin standard specific amidase activity. Amidase activity (substrate S-2251) of the plasmin standard was 0.033 o.u. of pNA released by 1 μg/ml of enzyme per 1 minute at 405 nm. Then the concentration of plasmin was

$$C = \frac{\Delta E_{405}\ o.u.}{N_{min}*0.033\ o.u./(\frac{\mu g}{ml}*\text{min})} = \frac{\Delta E_{405}}{N*0.033}$$ μg/ml, where

N_{min} — duration of measurement of plasmin activity in minutes;
ΔE405 o.u. — growth of the optical density in the cuvette during the measurement;
0.033 o.u./(μg/ml*min) — rate of p-NA release by 1 μg/ml of plasmin per 1 min.

Conclusion. The proposed methods to determine the activity of thrombin and plasmin made it possible to quantitatively calculate the rate of prothrombin activation in the lag period, the concentration and activity of thrombin based on the rate of fibrin and protofibrils formation as well as the activity and concentration of plasmin at the point of the complete clot dissolution,

the average rate of activation of plasminogen during the destruction of the clot structure and the time of formation and dissolution of the three-dimensional structure of the clot.

Key words: thrombin; plasmin; blood plasma coagulation; hemostasis; coagulation; fibrinolysis.

Acknowledgement. I am grateful to Prof. E.M. Makogonenko for supervising my research.

Author's contribution. A.V. Udovenko performed experiments, calculations and analysis of results and writing theses.

Founding. The research was financed by the project 0119U002512 of Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine.

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