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A fast and simple approach to the quantitative evaluation of fibrinogen coagulation

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Abstract

Fibrinogen is essential in the intrinsic and extrinsic blood coagulation process. Inhibition of fibrinogen aggregation could lead to anticoagulation effects. The availability of methods for easy quantitative evaluation of the coagulation process is critical to studying coagulation and its inhibition. A commonly used method is UV-visible absorbance (405 nm) detection by a micro-plate reader. However, because of the heterogeneous nature of the resulting mixture in a coagulation process, transmission-based optical measurements give large variations. Herein, a very simple and easy method is developed for the quantitative measurements of the coagulation process. The method was validated using three known thrombin inhibitors: 4-(2-aminoethyl) benzenesulfonyl fluoride (IC₅₀: 0.01 mM), p-amidinophenyl methanesulfonyl fluoride (IC₅₀: 0.18 mM) and PMSF (IC₅₀: 0.23 mM).

Keywords

Anti-coagulation; Blood; Coagulation; Fibrinogen; Fibrinogen clotting test; Thromin; Thrombin inhibitors

Introduction

Blood coagulation is an important process for the proper maintenance of the bleeding time. Fibrinogen aggregation is the last step in blood coagulation. (Mosesson 2005; Laki and Gladner 1964) In this process, thrombin catalyzes the conversion of soluble fibrinogen to white thread-like fibrin. (Scheraga and Laskowski 1957; Doolittle 1973) As a result, the fibrinogen-clotting test is a widely accepted method in determining the effect of anticoagulants. (Liu et al. 1979; Sano-Martins et al. 1994; Vermeylen et al. 1963) In the anticoagulation research area, the availability of an easy and quantitative method to measure coagulation is critical. Several methods, including a stopped-flow light scattering method, (Hantgan and Hermans 1979; Rossi et al. 1988; Casassa 1955; Kita et al. 2002; Bernocco et al. 2000) and a UV absorbance (405 nm) method (Ashour et al. 1987) have been reported. The light scattering method is based on the increase in light scattering (Huglin 1972) when

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fibrin is formed from fibrinogen, while the UV- visible method measures the absorbance of the solution at 405 nm. (Ashour et al. 1987). The light scattering method uses equipment not readily available in ordinary research laboratories and the absorbancy methods suffer from problems of inaccuracy and large variations due to the inhomogeneous nature of the fibrin solution/mixture. Herein, an easy optical method is developed. It can directly record the process of the fibrinogen coagulation by capturing images of the heterogeneous mixture. (Laura et al. 1980; Hsia et al. 1996). The amount of clotted fibrinogen can then be assessed using a computer software such as Image J. (Collins 2007; Rasband 1997; Abràmoff et al. 2004). The intensity of each sample is obtained by calculating the average intensity in unit areas to minimize errors in inhomogeneous solutions.

Materials and methods

All reagents were purchased from Sigma-Aldrich. 4-(2-Aminoethyl) benzenesulfonyl fluoride (AEBSF) stock solution (1 mM) was prepared in HCl (pH 5.1). PMSF stock solution (10 mM) was prepared in DMSO. p-Amidinophenyl methanesulfonyl fluoride (APMSF) stock solution (10 mM) was prepared in deionized water.

All the tests were performed in 50 mM Tris/HCl (pH: 7.4) buffer. Fibrinogen (2 μ M final concentration) was mixed with CaCl₂ (20 mM) in Tris/HCl buffer. Thrombin inhibitors (AEBSF: 6.4~100 μ M; PMSF: 0.1~1.5 mM; p-APMSF: 0.1~1.5 mM) were mixed with thrombin (0.2 IU/ml final concentration) in Tris/HCl buffer separately. Two mixtures were combined and incubated in a 96-well plate at 37 °C for 30 min. Images were captured up to 30 min in a dark room using a digital camera equipped with appropriate lenses. The largest circular area without a bubble or refraction in each well was selected for calculation.

Results and discussion

Coagulation processes were studied using commercially-available human fibrinogen with the addition of thrombin to trigger coagulation. To fully validate the method, we studied fibrinogen coagulation itself and in the presence of three known thrombin inhibitors [AEBSF (Hsia et al. 1996; Mintz 1993), p-APMSF (Laura et al. 1980) and PMSF (Hsia et al. 1996; Turini et al. 1969)] (Fig. 1). Specifically, different concentrations of inhibitors were incubated with the same amount of fibrinogen (2 μ M) in the presence of thrombin (0.2 IU/ml). A 96-well plate was used for the study. Images were captured at different times after mixing with thrombin. As shown in Fig. 2, an example of the appearance of fibrin at 0, 3, 6, 10, 15, and 30 min points. In each image, different concentrations of inhibitors were added to each well following the pattern described in Fig. 2g.

Quantitative determination of fibrin formation was based on the analysis of the optical density at each well using Image J (NIH), which is an image-processing program to analyze images through the calculation of light intensity in a specific area chosen based on the integral method. (Abràmoff et al. 2004; Collins 2007; Rasband 1997) Specifically for each well, an area without refraction was selected and then the light intensity was calculated and exported to a data processing program such as Microsoft Excel. Unit intensity was obtained by using whole intensity value divided by area. Fig. 3a shows an example of time-dependent coagulation of fibrinogen in the presence of AEBSF.

All three thrombin inhibitors showed time-dependent and concentration-dependent inhibition, as expected. IC₅₀ values were calculated by curve fitting in Prism5 software (Fig. 3b and Table 1). Standard deviations might due to the difference of light intensity and reflection when taking the photograph; the concentration range is also critical to experimental data. For example, the inhibition activity of p-APMSF is very sensitive

between 0.3 and 1 mM. All three inhibitors showed concentration dependent effect in the clotting test and all the results are reproducible (Supplementary Fig. 1).

There are several specific advantages of the method described.

First, the coagulation changes can be observed and analyzed in single photographs. This is a very easy and efficient process. Second, because all results from a plate are acquired at the same time, there is a high degree of consistency. Third, the method uses equipment readily available in a modern lab. In addition, because of the inhomogeneous nature of the fibrin solution/mixture, not all single-point determinations would be expected to give the same results in a light scattering experiment. However, with the image-capturing methods, we calculated the average intensity of a circular area with a fixed size for each well. The largest circular area without any bubble and refraction was selected in each well for calculation. Light intensity is average over the entire area, minimizing data fluctuation.

Conclusions

An optical density method has been developed for the detection of fibrinogen coagulation process. The method was validated by using known inhibitors of thrombin and by studying their effect on thrombin-mediated fibrinogen coagulation. The reproducibility was also good based on our triplicate results for this method.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Structures of three thrombin inhibitors.

Fig. 2. Images of clotting process at different time points in the presence of AEBSF. Concentration of AEBSF for six wells follows the pattern shown in “g.” The images were captured in a dark room at room temperature. g) Concentrations

a

b

Fig. 3. Inhibition curve of a) AEBSF (The inhibition curve is calculated by Image J software. The test is processed under 37°C, pH=7.4 Tris HCl buffer.). b) IC₅₀ determination for AEBSF

(The inhibition curve is calculated by GraphPad Prism 5. $Y = IF(X < X_0, Y_0, Plateau + (Y_0 - Plateau) * \exp(-K * (X - X_0)))$ IC₅₀ = Tau value)

Table 1

Calculated IC₅₀ values of AEBSF, *p*-APMSF and PMSF.

Inhibitors	IC ₅₀ ^a
AEBSF	0.01 ± 0.002 mM
<i>p</i> -APMSF	0.18 ± 0.05 mM
PMSF	0.23 ± 0.03 mM

^a Determinations of IC₅₀ values were based on triplicate results for AEBSF and PMSF and duplicate results for *p*-APMSF.