The Novel Assay Method for Thrombin by Weighing Fibrin Clot

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This study was performed to establish a simple and rapid method for measuring thrombin activity based on weight of fibrin clot formed. The new method was based on the weight measurement of fibrin clot after enzymatic reaction of thrombin with fibrinogen. The fibrin formation depended upon the activities of thrombin used, temperature, incubation time, and centrifugation time. The fibrin formation was increased proportionally up to 1.0 unit/ml of thrombin activity, 4.0 mg/ml of fibrinogen concentration, and 5 min of incubation time at 37℃. The fibrin clot formed was stable by centrifugation at 3,000×*g* for 5 min. This simple assay based on weight of fibrin after centrifugation would be useful for identifying natural food anticoagulants by inhibiting thrombin.

Key words – Thrombin, Assay, Fibrin formation

Blood coagulation is formed by a series of zymogen activations. A fibrin clot is formed by the interplay of the intrinsic, extrinsic, and final common pathways. The intrinsic pathway begins with the activation of Factor XII by contact with abnormal surfaces produced by injury. The extrinsic pathway is triggered by trauma, which activates Factor VII and releases a lipoprotein called tissue factor from blood vessels. This extrinsic clotting pathway and intrinsic one converge on a common sequence of final steps to form a fibrin clot [1,2]. Thrombin is central to the process of coagulation and monitoring its activity is a reliable indicator of the rate and extent of coagulation.

There are various methods to assay thrombin such as spectrophotometric [3,4], spectrofluorometric [5], enzyme-linked coagulation assay [6] and fiber optic evanescent waver sensor [7]. Most of the methods are based on the spectrophotometometric procedure using chromogenic or fluorescent substrates.

However, these methods are very complicated and not practical for routine use, or at least not economical. Also these methods are required the specific tools or machine. Here we report a simple, rapid, and economical method for measuring the thrombin activity, which is determined by the extent of fibrin formation. This novel assay method is not only an easy and rapid assay for measuring the thrombin activity but also suitable for analyses of many samples in a short time. That could be monitoring coagulant activity stimulated by thrombin and anticoagulant activity inhibiting thrombin.

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Materials and Methods

Materials

Fibrinogen and thrombin were from Sigma Chemical Co. All other reagents used were of analytical grade.

The novel assay method on thrombin activity

Thrombin activity was determined by the extent of fibrin formation. The assay was initiated by adding thrombin (1.0 U/ml) to fibrinogen solution (4 mg/ml) with 50 mM borate (pH 7.8) buffer. The total assay volume was 0.9 ml and reaction solution was incubated at 37℃ for 5 min. After the reaction, it was centrifuged at 3,000 x *g* for 5 min at room temperature. After centrifugation, the supernatant was discarded and the fibrin formed was estimated by measuring the weight of the precipitate. Fibrin formation (%) was expressed as fraction of fibrin formed from assay mixture, and the values were average of triple experiments.

$$\text{Fibrin formation (\%)} = (\text{weight of fibrin formed} / \text{weight of assay mixture}) \times 100$$

Results and Discussion

The thrombin activity was determined to the extent of fibrin formation using the novel assay method. Fig. 1 shows the effect of varying amount of thrombin on the fibrin formation. The fibrin formation was proportional to the concentration of thrombin and was linearly increased up to 1.0 unit/ml of thrombin used. Above that, it became

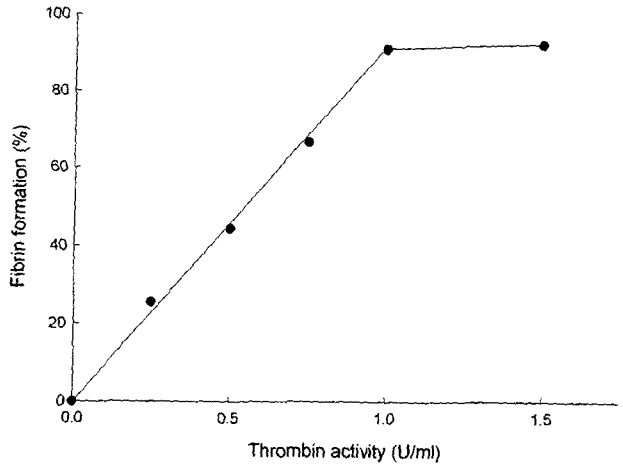

Fig. 1. The effect of thrombin activity on fibrin formation. The fibriniogen concentration was 5.0 mg/ml, and the reaction was performed at 37℃ for 5 min. The fibrin formation (%) was the fraction of fibrin formed from assay mixture and described in Method.

nonlinear indicating the presence of limiting factor. Thus the activity of thrombin used in the novel assay method conditions was 1.0 unit/ml.

Fig. 2 illustrates the effect of various concentration of fibrinogen, at a fixed amount of thrombin (1.0 unit/ml), on the fibrin formation. The amount of fibrinogen should be high enough not to be limiting factor for thrombin determination. As shown in Fig. 2, the fibrin formation was saturated above 4.0 mg/ml of fibrinogen used. And the fibrin formed above the fibrinogen concentration was stable during the measurement. The thrombin activity was assayed at various temperature ranging from 10℃ to 50℃. The thrombin activity was expressed as the extent of fibrin formation and it was not sensitive to incubation temperature (Fig. 3). At 15℃, fibrin clot was formed to 90% and above that temperature, the fibrin formation was retained as high as that value. Although it had a little difference, fibrin formation was achieved at the highest value at 37℃. Therefore, the incubation temperature of thrombin activity was determined to be 37℃.

Fig. 4 exhibits the incubation time dependency on thrombin activity. The fibrin formation was linear up to 10 min when the reaction was assayed as described in Methods. Therefore, the reaction time of thrombin was performed for 10 min.

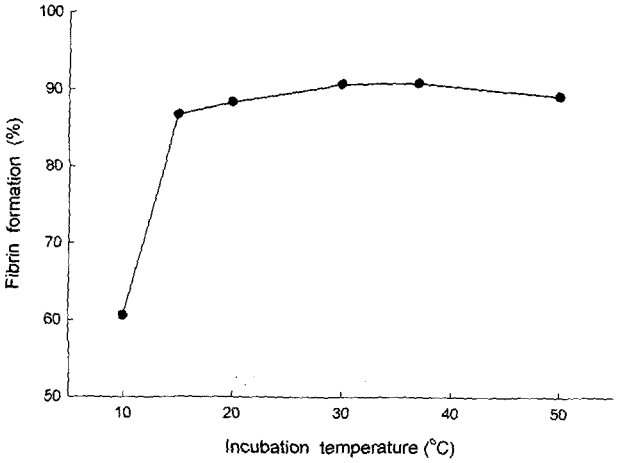

Fig. 3. The effect of temperature on fibrin formation. The thrombin activity used was 1.0 unit/ml, and the fibrinogen concentration was 4.0 mg/ml. The reaction was performed at various temperatures for 5 min.

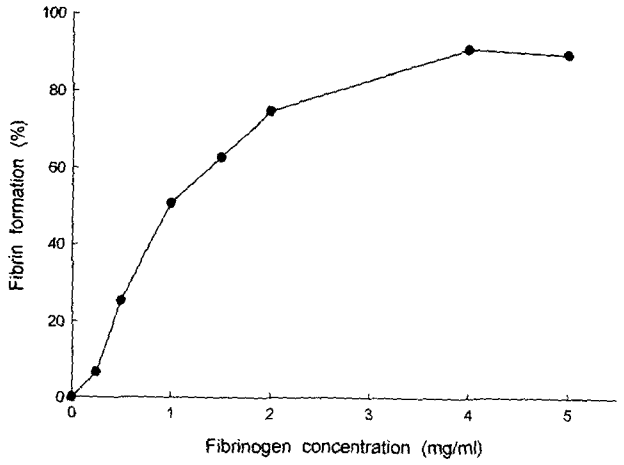

Fig. 2. The effect of fibrinogen concentration on fibrin formation.
The thrombin activity used was 1.0 unit/ml, and the reaction was performed at 37℃ for 5 min.

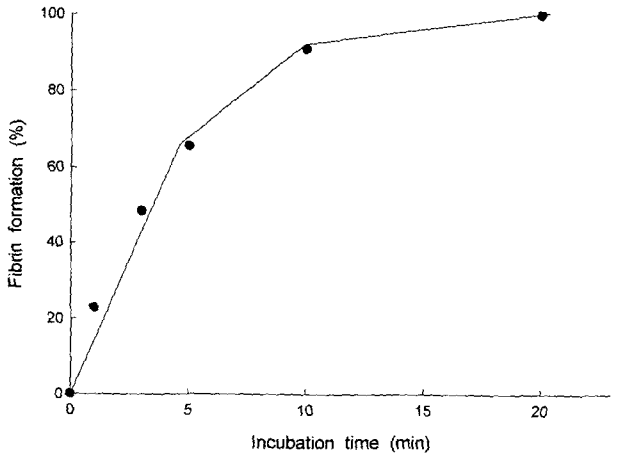

Fig. 4. The effect of incubation time on fibrin formation. The thrombin activity used was 1.0 unit/ml, and the fibrinogen concentration was 4.0 mg/ml. The reaction was performed ar 37℃ for various time.

Fig. 5 and Fig. 6 show the stability of fibrin clot formed by thrombin at the various centrifugation velocity and time after the reaction. The fibrin clot formed was very stable at the centrifugal force under 3,000×g. However, above that force the fibrin clot became unstable and at 8,000×g, the stability of fibrin clot was decreased to 50%. In the centrifugation time, the stability of fibrin clot was retained up to 5 min but was decreased to 77% during 7 min. Therefore, the optimum condition of the centrifugation force and time in the assay method was determined to be at 3,000×g for 5 min.

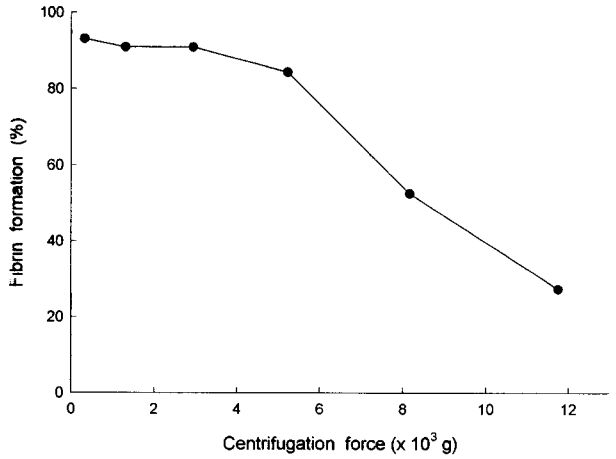

Fig. 5. The effect of centrifugal force on fibrin stability. The formed fibrin was centrifuged at various centrifugal forces for 5 min.

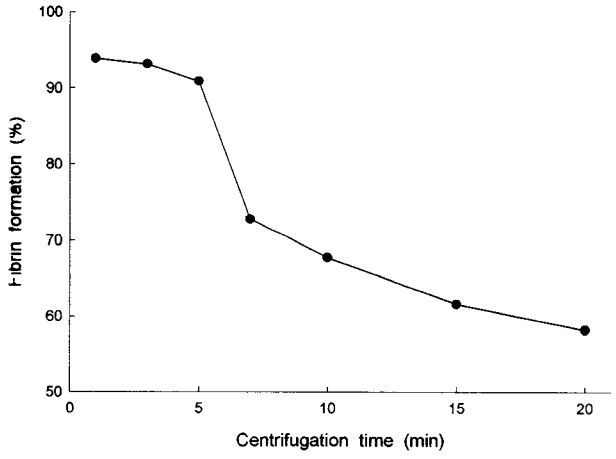

Fig. 6. The effect of centrifugation time on fibrin stability. The formed fibrin was centrifuged at 3,000×g for various times.

When natural products such as food materials were tested as an anticoagulant, the color containing in foods would interfere the spectrophotometric thrombin assay. Therefore, this new method based on weight of fibrin clot would be suitable for assaying anticoagulative natural products such as foods. This simple assay method for measuring thrombin activity has the merit of simplicity and convenience as well as low cost of operation.

Acknowledgement

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초록 : 피브린의 무게측정에 의한 새로운 트롬빈활성측정법

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본 연구는 피브린의 무게를 측정하는 방법에 의하여 간단하고 단순하게 트롬빈활성을 측정하는 방법을 확립하기 위하여 수행되었다. 이 새로운 트롬빈측정법은 트롬빈이 기질인 피브리노겐과 반응 후 생성된 피브린의 무게를 측정한다. 피브린의 생성은 사용한 트롬빈의 효소활성, 반응온도, 반응시간 및 원심분리의 시간에 의존하였다. 피브린의 생성은 37℃에서 트롬빈활성이 1.0 unit/ml 이내, 4.0 mg/ml의 피브리노겐 농도 및 반응시간이 5분이내에는 직선적으로 증가하였다. 그리고 생성된 피브린은 3,000×g에서 5분간 원심분리의 경우에 안정하였다. 이 새로운 피브린 측정법은 트롬빈을 저해하는 천연물질을 탐색하는데 유행하게 사용될 것으로 기대된다.