



Letter to the Editor

## Evaluation and Improvement of *in vitro* Detection Methods of Thrombin Inhibitor Activity

### 1. INTRODUCTION

Thrombin inhibitors are the main drugs for the prevention and treatment of thrombotic diseases. Although currently used thrombin inhibitors such as bivalirudin and argatroban show good therapeutic effects, the bleeding and poor oral bioavailability were reported to be their typical shortcomings [1,2]. Therefore, new thrombin inhibitors that can reduce these shortcomings are attracting more and more attention. At present, the chromogenic substrate method, Fibrinogen Clotting Time (FCT) assay method and spectrophotometry (Yang's method) are widely used in the preliminary screening stage of thrombin inhibitors. Compared with the other two methods, the chromogenic substrate method is widely used as a rapid and accurate method for assaying thrombin inhibitors activity. Meanwhile, ethylene diamine tetraacetic acid (EDTA) is added to the whole detection system, so the effect of divalent metal ions on the results can be eliminated [3]. However, the chromogenic substrate method is only suitable for the detection of inhibitors that can combine with the active site of thrombin, rather than for inhibitors that only combine with thrombin exosite-I. Since the chromogenic substrate method reflects the activity of thrombin by measuring the absorbance value, it is not suitable for detecting colored inhibitors (This problem also exists in Yang' method.). Although these shortcomings are perfectly avoided in the FCT assay method, the method still has fatal defects. The use of fibrin clotting time to characterize thrombin activity leads to certain requirements for ion concentration in the entire reaction system.

Yang et al. [4] proposed a method for assaying the activity of the thrombin inhibitor. The principle of this method is that the amount of fibrin clot formation is related to the activity of thrombin when the amount of fibrinogen is constant. Clot formation cause changes in absorbance at 405 nm and the inhibition rate of thrombin was calculated according to the following formula

$$Y\% = \frac{A_e - A_i}{A_e - A_o} \times 100$$

Y – Hydrolysate inhibition rate (%).

$A_e$  – Absorbance value without inhibitor.

$A_i$  – Absorbance value with inhibitor.

$A_o$  – Blank absorbance.

The method has been cited for 40 times and compensates for the shortcomings of the chromogenic substrate method, which can be used to detect both active site inhibitors and exosite-I inhibitors [5–7]. However, this method also showed some limits as followings.

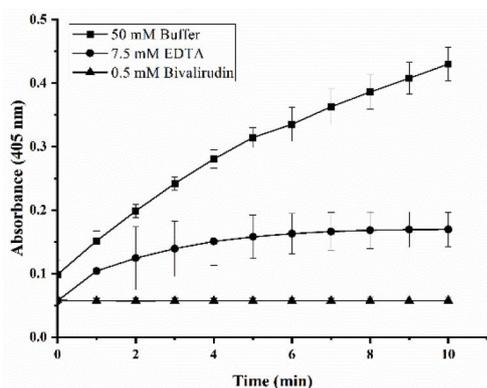
Firstly, there is a problem with the choice of absorbance. Yang's method finally determines the absorbance at 405 nm. However, the entire reaction system appears white, opaque at the time of final solidification, rather than yellow or other colors. Therefore, the reason for the difference in absorbance is the solidification of the solution leading to an increase in turbidity rather than a change in color. However, absorbance of 350 nm or 650 nm were chosen in the similar methods [8,9]. Secondly, there is no direct relationship between thrombin inhibitory activity and the value of absorbance. Yang believes that the higher the absorbance, the higher the thrombin activity and the lower the inhibitor activity; conversely, the lower the absorbance, the lower the thrombin activity and the higher the inhibitor activity. However, the magnitude of the absorbance value is related to the concentration of divalent metal ions. Although divalent metal ions such as  $Mg^{2+}$  and  $Fe^{2+}$  have no significant effect on the coagulation of fibrinogen and the absorbance value of the clot (only affect the formation of thrombus), the impact of  $Ca^{2+}$  and  $Zn^{2+}$  cannot be ignored [10–12]. Yang did not mention the concentration of  $Ca^{2+}$  and  $Zn^{2+}$ , only sodium ions were added to the buffer. Therefore, this method is prone to false positive results. In other words, samples with  $Ca^{2+}$  or  $Zn^{2+}$  chelating ability can be mistaken for thrombin inhibitors by using this method.

In order to confirm that there may be a false positive result and prove the significance of metal ions, the following experiments were carried out. The first experimental methods, buffers, calculation methods, etc. are the same as those proposed by Yang et al. [4]. In brief, 140  $\mu$ L fibrinogen with a concentration of 0.1% and 40  $\mu$ L sample were added into the plate wells (FEP101296, JET Biofil, China), incubated at 37°C for 5 min. 10  $\mu$ L thrombin (12 U/mL) was added to start the reaction and the absorbance at 405 nm was measured every minute for a total of 10 min. As for the samples, three kinds of samples have been set up, a positive control, the experimental group, and the negative control, they were 0.5 mM bivalirudin, 7.5 mM EDTA (same concentration as chromogenic substrate method) and 50 mM Tris-HCl buffer, respectively. Results were shown in Figure 1. The results showed that both bivalirudin and EDTA had good thrombin inhibitory activity. In fact, EDTA has no thrombin inhibitory activity, just a metal ion chelating agent. The results indicate that if the sample has the same metal chelating properties as EDTA, the sample may be mistaken for antithrombin activity according to this experimental method in the study of Yang etc.

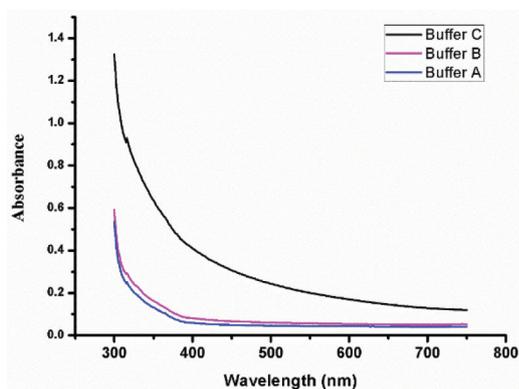
In order to demonstrate the effect of  $Ca^{2+}$  and  $Zn^{2+}$  on absorbance, the following experiment was carried out. Three kinds of buffer (pH = 7.4) were prepared for this experiment. Buffer A was 50 mM

Tris-HCl with 0.12 mM NaCl. Buffer B was 50 mM Tris-HCl with 0.12 mM NaCl and 2 mM  $\text{CaCl}_2$ . Buffer C was 50 mM Tris-HCl with 0.12 mM NaCl and 15  $\mu\text{M}$   $\text{ZnCl}_2$ . Fibrinogen (1 mg/mL) and thrombin (12 U/mL) were dissolved in three kinds of buffer, respectively. The method was conducted according to Yang's method [4], but the absorbance was determined at the wave length ranging from 350 to 750 nm. Results were shown in Figure 2. Although  $\text{Zn}^{2+}$  has a greater effect on fibrinogen turbidity than  $\text{Ca}^{2+}$ , both ions influence absorbance. The result is consistent with previous report [10].

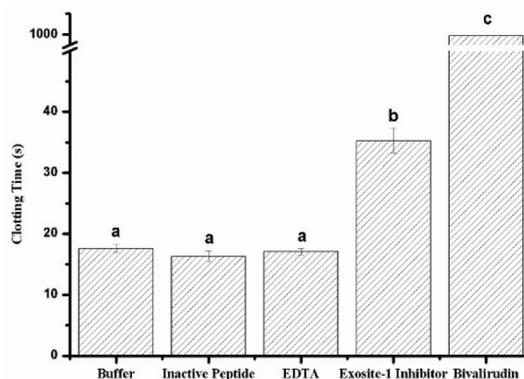
In order to eliminate the occurrence of false positives due to the influence of ions on the absorbance, the clotting time is measured to replace the absorbance at 405 nm. In brief, the fibrinogen and thrombin were all dissolved in 50 mM Tris-HCl buffer with 100 mM NaCl and 2 mM  $\text{CaCl}_2$ . The concentrations of fibrinogen and thrombin are 1 mg/mL and 10 U/mL, respectively. FCT assay was determined in accordance with references with minor but critical modifications [13]. As for as FCT, 200  $\mu\text{L}$  fibrinogen and 50  $\mu\text{L}$  buffer or sample were incubated for 60 s at 37°C, 50  $\mu\text{L}$  thrombin was added and the clotting time was measured with a coagulometer (MC4, Merlin, Germany). The clotting time of blank (50 mM Tris-HCl buffer), EDTA (7.5 mM), inactive peptide as negative control



**Figure 1** | Effect of different samples on absorbance. 0.5 mM bivalirudin ( $\blacktriangle$ ) was added as positive control, 7.5 mM EDTA ( $\bullet$ ) was added as metal ion chelating agent without antithrombin activity and 50 mM Tris-HCl buffer ( $\blacksquare$ ) was added as negative control.



**Figure 2** | Effect of different buffers on absorbance. Buffer A (blue) was 50 mM Tris-HCl with 0.12 mM NaCl. Buffer B (magenta) was 50 mM Tris-HCl with 0.12 mM NaCl and 2 mM  $\text{CaCl}_2$ . Buffer C (black) was 50 mM Tris-HCl with 0.12 mM NaCl and 15  $\mu\text{M}$   $\text{ZnCl}_2$ .



**Figure 3** | Fibrinogen clotting time of different samples. Thrombin exosite-I inhibitor and bivalirudin (bivalent direct thrombin inhibitor) have significant difference compare with buffer (blank) but inactive peptide (negative control) and EDTA are not.

(1.25 mM), thrombin exosite-I inhibitor (0.6 mM) and bivalirudin (0.6 mM) were measured. The detection index changes from absorbance to solidification time, which can effectively avoid the influence of  $\text{Zn}^{2+}$  on the experimental results. Meanwhile, adding enough  $\text{Ca}^{2+}$  to the reaction system can avoid the false positive result caused by the difference in  $\text{Ca}^{2+}$  concentration. As shown in Figure 3, the FCT of thrombin exosite-I inhibitor and bivalirudin shows significant difference compared with control, but inactive peptide and EDTA are not. Calculate the Relative Standard Deviation (RSD) value of the test results of the two methods, the original method RSD value exceeds 10% (RSD value was 13.3%), and the new method RSD value is <5% (RSD value was 3.0%). When the thrombin activity increased to 12 U/mL, the RSD value was 1.9%. When the thrombin activity decreased to 6 U/mL, the RSD value was 9.2%.

## 2. CONCLUSION

Based on the analysis above, an assay method of the inhibitory activity against thrombin was established as following. The reaction system was operated in coagulometer at 37°C, and the pH 7.4. The concentration of fibrinogen and thrombin was 0.1% and 10 U/mL, respectively. 200  $\mu\text{L}$  fibrinogen and 50  $\mu\text{L}$  Tris-HCl buffer (blank) or sample were added into automated coagulation cup with a magnetic bead and incubated for 60 s. Then, 50  $\mu\text{L}$  thrombin was added into automated coagulation cup and the clotting was measured. Tris-HCl buffer (50 mM Tris, 0.1 M NaCl and 2 mM  $\text{CaCl}_2$ , pH = 7.4) was used in the present method. The concentration of the sample is recommended to be within 5 mM, not to exceed 10 mM and the thrombin concentration cannot lower than 10 U/mL. Compared with Yang's method, although the method in our study cannot calculate the thrombin inhibition rate of inhibitors, it can still be used as a rapid detection method for large-scale screening and characterization of inhibitors. The improved method no longer uses absorbance as the detection index and adds enough necessary ions to the entire system to avoid the influence of the difference in metal ion content on the results. Meanwhile, a lower RSD value indicates that it has better repeatability than Yang's method. Compared with the chromogenic substrate method, this method has a wider detection range and can also accurately characterize thrombin

inhibitors which only inhibit thrombin exosite-I. Therefore, this method can be used in the extensive screening and preliminary characterization of thrombin inhibitors.

## CONFLICTS OF INTEREST

The authors declare they have no conflicts of interest.

## ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

## REFERENCES

- [1] Scatena R. Bivalirudin: a new generation antithrombotic drug. *Expert Opin Investig Drugs* 2000;9:1119–27.
- [2] Steinmetzer T, Stürzebecher J. Progress in the development of synthetic thrombin inhibitors as new orally active anticoagulants. *Curr Med Chem* 2004;11:2297–321.
- [3] Xu S, Fan F, Liu H, Cheng S, Tu M, Du M. Novel anticoagulant peptide from lactoferrin binding thrombin at the active site and exosite-I. *J Agric Food Chem* 2020;68:3132–9.
- [4] Yang WG, Wang Z, Xu SY. A new method for determination of antithrombotic activity of egg white protein hydrolysate by microplate reader. *Chinese Chem Lett* 2007;18:449–51.
- [5] Zhang SB. In vitro antithrombotic activities of peanut protein hydrolysates. *Food Chem* 2016;202:1–8.
- [6] Yu Z, Liu B, Zhao W, Yin Y, Liu J, Chen F. Primary and secondary structure of novel ACE-inhibitory peptides from egg white protein. *Food Chem* 2012;133:315–22.
- [7] Nasri R, Amor IB, Bougateg A, Nedjar-Arroume N, Dhulster P, Gargouri J, et al. Anticoagulant activities of goby muscle protein hydrolysates. *Food Chem* 2012;133:835–41.
- [8] Park R, Ping L, Song J, Hong SY, Choi TY, Choi JR, et al. Fibrinogen residue  $\gamma$ Ala341 is necessary for calcium binding and 'A-a' interactions. *Thromb Haemost* 2012;107:875–83.
- [9] Jablonka W, Kotsyfakis M, Mizurini DM, Monteiro RQ, Lukszo J, Drake SK, et al. Identification and mechanistic analysis of a novel tick-derived inhibitor of thrombin. *PLoS One* 2015;10:e0133991.
- [10] Marx G. Mechanism of fibrin coagulation based on selective, cation-driven, protofibril association. *Biopolymers* 1988;27:763–74.
- [11] Tang X, Zhang Z, Fang M, Han Y, Wang G, Wang S, et al. Transferrin plays a central role in coagulation balance by interacting with clotting factors. *Cell Res* 2019;30:119–32.
- [12] Mussoni L, Sironi L, Tedeschi L, Calvio AM, Colli S, Tremoli E. Magnesium inhibits arterial thrombi after vascular injury in rat: *in vivo* impairment of coagulation. *Thromb Haemost* 2001;86:1292–5.
- [13] Koh CY, Kazimirova M, Trimnell A, Takac P, Labuda M, Nuttall PA, et al. Variegin, a novel fast and tight binding thrombin inhibitor from the tropical bont tick. *J Biol Chem* 2007;282:29101–13.

Hanxiong Liu<sup>1</sup>, Shuzhen Cheng<sup>1,2</sup>, Zhenyu Wang<sup>1</sup>, Ming Du<sup>1,\*</sup>

<sup>1</sup>School of Food Science and Technology, National Engineering Research Center of Seafood, Dalian Polytechnic University, Dalian 116034, China

<sup>2</sup>Beijing Advanced Innovation Center for Food Nutrition and Human Health, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China

\*Corresponding author. Email: [duming@dlpu.edu.cn](mailto:duming@dlpu.edu.cn)

Peer review under responsibility of the *International Association of Dietetic Nutrition and Safety*