Preparation of thrombin from Human Cryo-removed Plasma using Russell's Viper Venom Factor X Activator (RVV-X)

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***Abstract*—Thrombin was prepared from human cryo-removed plasma (CRP) following activation with calcium chloride (condition A), with RVV-X for 2 hr (B), or without incubation time (C). A 36 kDa thrombin band appeared in all conditions with total protein concentration of 0.076, 0.063 and 0.065 mg/dL, respectively. The prepared thrombin solution (diluted 1:50) showed the results of thrombin time at 15, 8 and 9 seconds, respectively. Thrombin prepared RVV-X activation (B) have faster clotting time than standard condition despite reduction in incubation time (C).**

***Index Terms*— Cryo-removed plasma, Russell's viper venom factor X activator, Thrombin preparation.**

I. Introduction

Thrombin (factor IIa), a serine protease that converts fibrinogen into fibrin in blood coagulation, has plays a crucial role in hemostasis and thrombosis. Thrombin is activated from its zymogen, prothrombin, at the site of tissue injury by Factor Xa and its cofactor Factor Va in the presence of phospholipid membrane and calcium [1-2]. Thrombin is a common hemostatic drug used in surgical practice for over 100 years because of its simplicity and efficacy [3]. Thrombin is present in fibrin glue (FG), a plasma-derived product that composes of fibrinogen and thrombin, which use for reduce blood loss in many types of surgery [4-7, 11]. Thrombin products also has been used in clinical practice for coagulation test thrombin time (TT) [8]. Thrombin is isolated from plasma obtained from bovine or human sources [9]. The plasma is processed through a series of separation and filtration steps followed by incubation of the solution with calcium chloride to isolate and activate prothrombin to thrombin [10], it is also well known that prothrombin can be activated by some components of snake venom to yield thrombin [11]. The solution subsequently undergoes ultrafiltration, vapor heat treatment, solvent-detergent treatment, sterile filtration and freeze-drying [10]. The bovine thrombin added the additional risk of contamination with bovine spongiform encephalopathy (BSE) and immunomediated coagulopathy because of antibodies to bovine Factor V reacting with human Factor V [9].

The National Blood Centre of the Thai Red Cross Society (NBC-TRCS) had produced FG product and dispensed to the hospitals over the country since 1999. Unfortunately, the plant had to stop FG production in 2010 due to discontinued supply of thrombin from foreign supplier.

We have purified a factor X activator from Russell viper (*Daboia russelli siamensis)* venom (RVV-X) at the Snake Bite and Venom Research Unit, Faculty of Medicine, Chulalongkorn University [12]. These report aims demonstrate a novel method of preparation of thrombin from CRP using RVV-X to activate prothrombin. This production method could provide a sustainable supply of human thrombin for clinical or laboratory use.

II. Materials and Methods

A. Cryo-removed plasma (CRP)

CRP, the plasma from which cryoprecipitate has been removed, was obtained from Blood Components Production Section of NBC-TRCS.

CRP was prepared from slowly thawed fresh frozen plasma overnight at 4°C and centrifuged to separate the plasma from the insoluble cryoprecipitate. The insoluble cryoprecipitate was removed and the remaining plasma is refrozen.

B. RVV-X preparation

Russell viper venom factor X activator (RVV-X) was prepared from crude venom from *Daboia russelli siamensis* using sequential column chromatography [12]. The specific Factor Xa activity was 1.240 nkat/ng.

C. Thrombin Preparation

Thrombin was prepared by a modification procedure from Human Blood Coagulation, Hemostasis and Thrombosis (R. Biggs, Ed). Blackwell, Oxford. (1972) [13]. The starting material, 100 mL of CRP, were diluted to 1000 mL with distilled water, adjusted pH to 5.3 with 2% acetic acid and centrifuged. The precipitate was dissolved in 25 mL of 0.85% sodium chloride and pH was adjusted to 7.0 with 2% sodium carbonate. This was followed by the addition of 3 mL of 0.25 M calcium chloride with or without RVV-X 50 μg and stood for 2 hours for full thrombin formation or without incubation time. The coagulated fibrin was removed and acetone was added to thrombin crude solution (volume 1:1) at room temperature. The solution was centrifuged and separated the precipitate. The precipitate was extracted with 10 mL 0.85% sodium chloride and centrifuged. The supernatant was collected as thrombin solution and stored below temperature -20°C.

Thrombin was prepared by the procedure described above comparing 3 different conditions as shown in Table I. All prepared thrombin were preliminary tested for their activities

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by observation of fibrin clot formation after mixed with cryoprecipitate obtained from Blood Components Production Section of NBC-TRCS, and then further analyzed by other methods for their properties.

TABLE I: CONDITIONS USED FOR THROMBIN PREPARATION IN THE STUDY.

Condition	Prothrombin conversion related factors		
	0.25 M Calcium chloride (mL)	RVV-X (µg)	Conversion time (hrs)
A	3	-	2
B	3	50	2
C	3	50	-

D. Molecular Weight and Protein Patterns

The molecular weight (MW) of prepared thrombin was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using standard protein molecular weight markers.

E. Total Protein Assays

Kjeldahl method was used for total protein assays of prepared thrombin as described by AOAC. [14]

F. Thrombin Time (TT)

Prepared thrombin was diluted to 1:50 with distilled water. One hundred microliters of diluted thrombin were added to 100 µL fresh plasma in the test tube at 37°C. The tube was gently shaken and tilted the tube back and forth. The time taken for the first appearance of a fibrin clot was recorded.

III. RESULTS AND DISCUSSION

A. Thrombin Preparation

Thrombin was prepared from CRP, which is rich in prothrombin complex. To assess the usefulness of RVV-X, we compared 3 preparation conditions as shown in Table I.

From starting volume of CRP 100 mL, we obtained about 10 mL thrombin solution in each preparation batch. The solution was slightly turbid with some small particles due to no filtration step in the process. Thrombin solution was kept in the refrigerator at temperature below -20°C for its stability before the properties were tested.

The preliminary test of all preparation batches showed fibrin clot formation after mixed prepared thrombin with cryoprecipitate which contains fibrinogen. This reaction confirmed that there was thrombin in all prepared solutions.

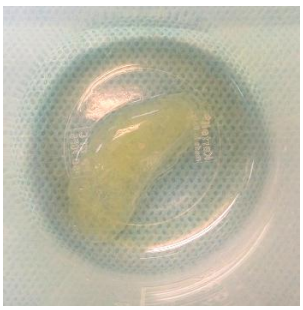

Fig. 1. Fibrin clot formation after mixed prepared thrombin with cryoprecipitate.

B. Molecular Weight and Protein Patterns

The MW of prepared thrombin was determined by SDS-PAGE using standard protein molecular weight markers (Figures 2).

CRP contains several protein including 72 kDa prothrombin, but not 36 kDa thrombin (lane D) [15-16]. Upon activation with calcium chloride (A) or RVV-X (lane B and C), the 36 kDa thrombin band appeared.

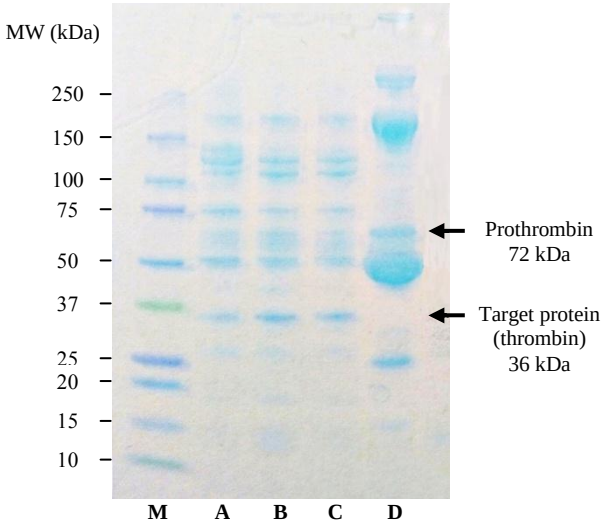

Fig. 2. SDS-PAGE of produced thrombin prepared from various conditions. Lane M: molecular weight markers; Lane A: condition A; Lane B: condition B; Lane C: condition C; Lane D: CRP supernatant before thrombin conversion

It was noticed that thrombin bands on the digest of condition B and condition C were more obvious than of condition A, this may because there were RVV-X used in the preparation process, which resulted in better prothrombin conversion reactions. In addition, thrombin band on the digest of condition B was more obvious than of condition C, this may be due to conversion time 2 hours gave completely prothrombin conversion reaction compare to no conversion time used.

C. Total Protein Assays

The results in Table II showed that total protein number in prepared thrombin from condition A, B and C are 0.076, 0.063 and 0.065 g/dL, respectively. However, the bigger total protein number does not reflect that it has more thrombin than the other samples due to several proteins also included in the prepared thrombin, as we can see in the SDS-PAGE result.

TABLE II: Results of total protein assays by Kjeldahl method and thrombin time testing.

Condition	Total protein (g/dL)	Thrombin time (sec)
A	0.076	15.0
B	0.063	8.0
C	0.065	9.0

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D.Thrombin Time (TT)

Thrombin time was detected after mixing of diluted thrombin (1:50) with fresh plasma. Thrombin time results of prepared thrombin from condition A, B and C were 15, 8 and 9 seconds, respectively. Thrombin prepared from condition B and C have similar numbers of thrombin time and have faster clotting time than condition A. This is because there were RVV-X presented in the preparation process that resulted in better prothrombin conversion reactions. Condition C maybe more convenient than condition B to prepare thrombin due to no conversion time 2 hours needed.

IV. Conclusion

We have succeeded in preparation of thrombin from CRP using RVV-X to improve product yield. Thrombin prepared from the process has MW about 36 kDa and give a satisfactory fibrin clot formation.

The prepared thrombin should be further determined by the coagulator analyzer to prove its actual activity in National Institutes of Health (NIH) units. Additionally, it should be purify with suitable affinity chromatography for higher purity and potency.

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