# II. MATERIALS AND METHODS

## A. Plasma preparation

Plasma was obtained by centrifugation of donated CPD-blood from Red Cross Society, Chiang Mai ($10^{th}$) at 4 °C, 3,000 rpm (KOKUSAN model H103HS) for 10 minutes.

## B. Methods

### PART I

### 1. Fibrinogen preparation

### 1.1 Fibrinogen preparation by cryoprecipitation

Plasma was diluted to 5 g/dl of protein with 0.85% sodium chloride (0.85% NaCl) and 30 ml of diluted plasma was used for cryoprecipitation (AABB, 1985). Fibrinogen, FV, FVIII and FXIII were not dissolved after frozen plasma was thawed at 4 °C. Diluted plasma was frozen at the temperature below –20 °C for 18-24 hours. The frozen diluted plasma was thawed at 4 °C for 6-10 hours. The thawed plasma was centrifuged at 3,000 rpm (KOKUSAN model H103HS) for 10 minutes at 4 °C. The supernatant was removed by using a pipette, leaving about 2 ml of the precipitate for further study and other determinations.

### 1.2 Fibrinogen preparation by repeat cryoprecipitation

Fibrinogen preparation by repeat cryoprecipitation was modified form AABB (1985) and Saltz R, *et al.* (1991). Plasma was diluted to 5 g/dl of protein with 0.85% NaCl. Thirty milliliters of diluted plasma was frozen at the temperature below –20 °C for 18-24 hours. The frozen diluted plasma was thawed at 4 °C for 6-10 hours. The thawed

plasma was refrozen again at temperature below –20 $^0$C for 18-24. The refrozen diluted plasma was thawed at 4 $^0$C for 6-10 hours. The thawed plasma was centrifuged at 3,000 rpm for 10 minutes at 4 $^0$C. The supernatant was removed by using a pipette, leaving about 2 ml of the precipitate for further study and other determinations.

1.3 **Fibrinogen preparation by saturated ammonium sulfate precipitation**

Fibrinogen preparation by saturated ammonium sulfate salting-out precipitation was modified from Siedentop KH, *et al.* (1986) and Park MS, *et al.* (1993). Plasma was diluted to 5 g/dl of protein with 0.85% NaCl. Thirty milliliters of diluted plasma was precipitated by 10.5 ml of saturated ammonium sulfate [0.35 ml $(NH_4)_2SO_4$/1 ml diluted plasma] and the mixture was centrifuged at 3,000 rpm for 10 minutes at 4 $^0$C. The supernatant was removed by using a pipette, leaving about 2 ml of the precipitate for further study and other determinations.

1.4 **Fibrinogen preparation by saturated ammonium sulfate precipitation and followed by cryoprecipitation**

Fibrinogen preparation by saturated ammonium sulfate, salting-out, precipitation and followed by cryoprecipitation was modified from AABB (1985), Siedentop KH, *et al.* (1986) and Park MS, *et al.* (1993). Plasma was diluted to 5 g/dl of protein with 0.85% NaCl. Thirty milliliters of diluted plasma was precipitated by 10.5 ml of saturated ammonium sulfate [0.35 ml $(NH_4)_2SO_4$/1 ml diluted plasma]. The mixture was frozen at the temperature below –20 $^0$C for 18-24 hours. The frozen mixture was thawed at 4 $^0$C for 6-10 hours. The thawed mixture was centrifuged at 3,000 rpm for 10 minutes at 4 $^0$C. The supernatant was removed by using a pipette, leaving about 2 ml of the precipitate for further study and other determinations.

1.5 **Fibrinogen preparation by absolute ethanol precipitation**

Fibrinogen preparation by absolute ethanol precipitation was modified from Park JJ, *et al.* (1997). Plasma was diluted to 5 g/dl of protein with 0.85% NaCl. Thirty milliliters of diluted plasma was precipitated by 6 ml of absolute ethanol (0.2 ml absolute ethanol/1 ml diluted plasma) at 4 $^{0}$C in icebath and stood at 0 $^{0}$C for 30 minutes. The mixture was centrifuged at 3,000 rpm for 10 minutes at 4 $^{0}$C. The supernatant was removed by using a pipette, leaving about 2 ml of the precipitate for further study and other determinations.

1.6 **Fibrinogen preparation by absolute ethanol precipitation and followed by cryoprecipitation**

Fibrinogen preparation by absolute ethanol precipitation and followed by cryoprecipitation was modified from AABB (1985) and Park JJ, *et al.* (1997). Plasma was diluted to 5 g/dl of protein with 0.85% NaCl. Thirty milliliters of diluted plasma was precipitated by 6 ml of absolute ethanol (0.2 ml absolute ethanol/1 ml diluted plasma) at 4 $^{0}$C in icebath and stood at 0 $^{0}$C for 30 minutes. The mixture was frozen at the temperature below –20 $^{0}$C for 18-24 hours. The frozen mixture was thawed at 4 $^{0}$C for 6-10 hours. The thawed mixture was centrifuged at 3,000 rpm for 10 minutes at 4 $^{0}$C. The supernatant was removed by using a pipette, leaving about 2 ml of the precipitate for further study and other determinations.

1.7 **Fibrinogen preparation by 10% ethanol precipitation**

Fibrinogen preparation by 10% ethanol precipitation was modified from Burnouf-Rodosevich M, *et al.* (1990). Plasma was diluted to 5 g/dl of protein with 0.85% NaCl. Twenty milliliters of diluted plasma was precipitated by 20 ml of 10% ethanol (1:1) at 4 $^{0}$C in icebath. The mixture was left to settle the precipitate at 4 $^{0}$C for 6 hours. The mixture was centrifuged at 3,000 rpm for 10 minutes at 4 $^{0}$C.

The supernatant was removed by using a pipette, leaving about 2 ml of the precipitate and incubated in 37 $^{0}$C waterbath about 10 minutes for further study and other determinations.

### 1.8 Fibrinogen preparation by 10% ethanol precipitation and followed by cryoprecipitation

Fibrinogen preparation by 10% ethanol precipitation and followed by cryoprecipitation was modified from AABB (1985) and Burnouf-Rodosevich M, *et al.* (1990). Plasma was diluted to 5 g/dl of protein with 0.85% NaCl. Twenty milliliters of diluted plasma was precipitated by 20 ml of 10% ethanol (1:1) at 4 $^{0}$C in icebath. The mixture was left to settle the precipitate at 4 $^{0}$C for 6 hours. The mixture was frozen at the temperature below –20 $^{0}$C for 18-24 hours. The frozen mixture was thawed at 4 $^{0}$C for 6-10 hours. The mixture was centrifuged at 3,000 rpm for 10 minutes at 4 $^{0}$C. The supernatant was removed by using a pipette, leaving about 2 ml of the precipitate and incubated in 37 $^{0}$C waterbath about 10 minutes for further study and other determinations.

### 1.9 Fibrinogen preparation by polyethylene glycol precipitation

Fibrinogen preparation by polyethylene glycol precipitation was modified from Silberstein LE, *et al.* (1988). Plasma was diluted to 5 g/dl of protein with 0.85% NaCl. Thirty milliliters of diluted plasma was precipitated by 15 ml of 2, 4, 10, 15 and 30% polyethylene glycol 8000 (PEG8000) at 4 $^{0}$C in icebath. The mixtures were centrifuged at 3,000 rpm for 10 minutes at 4 $^{0}$C. The supernatant was removed by using a pipette, leaving about 2 ml of the precipitate and incubated in 37 $^{0}$C waterbath about 10 minutes for further study and other determinations.

### 1.10 Fibrinogen preparation by polyethylene glycol precipitation and followed by cryoprecipitation

Fibrinogen preparation by polyethylene glycol precipitation was modified from AABB (1985) and Silberstein LE, *et al.* (1988). Plasma was diluted to 5 g/dl of protein with 0.85% NaCl. Thirty milliliters of diluted plasma was precipitated by 15 ml of 2, 4, 10, 15 and 30% polyethylene glycol 8000 (PEG8000) at 4 $^{0}$C in icebath. The mixture was frozen at the temperature below –20 $^{0}$C for 18-24 hours. The frozen mixture was thawed at 4 $^{0}$C for 6-10 hours. The mixture was centrifuged at 3,000 rpm for 10 minutes at 4 $^{0}$C. The supernatant was removed by using a pipette, leaving about 2 ml of the precipitate and incubated in 37 $^{0}$C waterbath about 10 minutes for further study and other determinations.

### 2. Determination of protein

Protein in plasma and all of the fibrinogen solutions from precipitation were determined by the Biuret method (Doumas BT, *et al.* 1981). Cupric ion is formed form complexes with the nitrogen atoms of the peptide linkages of protein. These complexes are reacted with the Biuret (carbamylurea, $NH_2CONHCONH_2$) reagent in a highly alkaline solution to form a violet-colored condensation product having a maximal absorption at 540 nm. One hundred microliters of plasma or fibrinogen solution was added into 4 ml of Biuret's solution. One hundred microliters of distilled water and 8 g/dl of protein standard (Sigma Diagnostics, Inc., Cat. No. 540-10) were added into 4 ml of Biuret's solution as blank and standard respectively. It was stood at room temperature for 30 minutes. The absorbance was read at 540 nm against blank. Protein in plasma and all of fibrinogen solutions were calculated as follows.

$$\text{Protein (g/dl)} = \frac{\text{Absorbance of unknown} \times \text{Concentration of standard}}{\text{Absorbance of standard}}$$

3. **Determination of albumin**

Albumin in plasma and all of the fibrinogen solutions from precipitation were determined by Bromcresol green method (Doumas BT, et *al.*, 1971). Albumin and Bromcresol Green (BCG) are allowed to bind at pH 4.2. At this pH, albumin acts as a cation to bind with the anionic dye. Absorption peak of the BCG-albumin complex is determined at 630 nm. Twenty microliters of plasma or fibrinogen solution was added into 4 ml of working BCG reagent. Twenty microliters of distilled water and 5 g/dl of albumin standard (Sigma Diagnostics, Inc., Cat. No. 540-10) were added into 4 ml of working BCG as blank and standard respectively. It was stood at room temperature for 10 minutes. The absorbance was read at 630 nm against blank. Albumin in plasma and all of fibrinogen solutions were calculated as follows.

$$\text{Albumin (g/dl)} = \frac{\text{Absorbance of unknown} \times \text{Concentration of standard}}{\text{Absorbance of standard}}$$

4. **Determination of fibrinogen concentration**

The fibrinogen concentration in plasma and all of the fibrinogen solutions from precipitation was determined by modified thrombin time method (Harmenning DM, 1997) and Ratnoff's method (Ratnoff OD and Menzie C, 1951).

4.1 **Modified thrombin time method**

This method is recommended for determination of the clottable fibrinogen level. Thrombin is added to 1:10 diluted plasma and catalyzes the conversion of fibrinogen to fibrin. The time of clot formation of plasma was compared with

the results of a fibrinogen reference. Its concentration is invert to the time of clot formation.

Fibrinogen reference (Sigma Diagnostics, Inc., Cat. No. 886-10) dilution was as follows.

| **Dilution** | **Fibrinogen reference (ml)** | **Imidazole buffer (ml)** | **Dilution factor** |
|---|---|---|---|
| 1:5 | 0.5 | 2.0 | 2 |
| 1:10 | 0.5 of 1:5 dilution | 0.5 | 1 |

The 1:10 dilution of the fibrinogen reference defines as 245 mg/dl. The values of fibrinogen in all other dilutions are calculated as follows:

Fibrinogen (mg/dl) = assayed value of fibrinogen reference (mg/dl) x dilution factor

Fibrinogen calibration curve results were plotted on graph paper or on 2-cycle log x log graph paper with fibrinogen concentration (mg/dl) on the X-axis and clotting time (seconds) on the Y-axis. The curve connecting the point is usually curvilinear.

Sample assay was as followed. Plasma, all of the fibrinogen solutions from precipitations and cryoprecipitate from Thai Red Cross Society were prepared by diluting them to 1:10 with imidazole buffer in plastic tubes. One hundred microliters of diluted plasma, diluted fibrinogen solutions or diluted cryoprecipitate prepared by Thai Red Cross Society was pipetted into cuvette. The cuvette was putted into the fibrinometer (Behring Fibrintimer II) and incubated for 60 seconds. Fifty microliters of thrombin reagent (Sigma Diagnostic, Inc., Cat. No. 886-12) was added into the cuvette. The fibrinometer was started to record the time until the fibrin is formed.

4.2 **Ratnoff's method**

Fibrinogen in plasma and all of the fibrinogen solutions from precipitation were determined by Ratnoff's method. Fibrinogen was formed fibrin clot by thrombin, in the presence of crushed glass. Sufficient thrombin was used to shorten the time necessary for complete clotting to occur. The fibrin adhered to the crushed glass and could be separated and washed by centrifugation. An amount of fibrin adherent to the crushed glass was determined by measuring its tyrosine-like activity by modifications of Wu's method by using fibrinogen reference standard substituted the standard solution of tyrosine.

One milliliter of distilled water, 1 ml of 10% sodium hydroxide (10% NaOH), 3 ml of 20% sodium carbonate (20% $Na_2CO_3$) and 1 ml of phenol were added into the 16x150 mm test tube as blank. One milliliter of color solution was diluted with 3 ml of distilled water. Ten of 0.5 mm in diameter Pyrex glass beads and 50 µl of 1140 NIH units/ml thrombin (Sigma Chemical Co, Cat. No. T-9000) were added in the 16x150 mm test tube. One hundred microliters of fibrinogen standard (Sigma Diagnostics, Inc., Cat. No. 886-10), plasma, all of the fibrinogen solution from precipitation and cryoprecipitate prepared by Thai Red Cross Society were added as standard and test, respectively. They were agitated and stood at room temperature for 10 minutes. Ten milliliters of 0.85% NaCl was added into these test tubes and centrifuged at 2000 rpm for 5 minutes. The supernatant was discarded. The precipitate was washed 2 times by 0.85% NaCl. The supernatant of the last time was removed completely. One milliliter of 10% NaOH was added. The test tubes was incubated in boiling waterbath for 10 minutes and kept the waterbath cover during this time. After leaving the test tubes cooled to room temperature, 7 ml of distilled water, 3 ml of 20% $Na_2CO_3$ and 1 ml of phenol was added. It was stood at room temperature for 30 minutes. One milliliter of color solution was diluted with 3 ml of

distilled water. The intensity of color was read against blank at 650 nm. The fibrinogen concentration in plasma and all of the fibrinogen solutions were calculated as follows.

$$\text{Fibrinogen (mg/dl)} = \frac{\text{Absorbance of unknown} \times \text{Concentration of standard}}{\text{Absorbance of standard}}$$

5. **Determination of the quality of fibrinogen**

The quality of fibrinogen in plasma and all of the fibrinogen solutions from precipitation were determined by thrombin time method (Fritsma GA, 1995). Fibrinogen is converted to fibrin by adding thrombin to citrated-plasma. The thrombin time is prolonged when the fibrinogen concentration is less than 100 mg/dl or greater than 400 mg/dl; when there is an abnormality of the functional integrity of fibrinogen; or when inhibitors of the reactions are presents. Plasma and all of the fibrinogen solutions were diluted into 1:10 by 0.85% NaCl. One hundred microliters of diluted plasma or diluted fibrinogen solutions was pipetted into cuvette and the cuvette was putted into the fibrinometer. One hundred microliters of the 5 NIH units/ml of thrombin (Sigma Chemical CO., Cat. No. T-9135) was added into the cuvette. The fibrinometer was started to record the time until the fibrin is formed.

6. **Determination of the quantity and quality of factor XIII**

The quantity and quality of factor XIII in plasma and all of the fibrinogen solutions from precipitation were determined by urea solubility test, the screening test for factor XIII deficiency (Harmenning PD, 1987). The stability of fibrin clot depends on factor XIII, which is formed covalent bond to crosslinked fibrin monomers by transamidation. In factor XIII deficiency, fibrin polymer, which has

hydrogen bond, will dissolved in 5 M urea. Two methods of urea solubility test were used in this study. The test was performed in duplicate.

The first method (Sirridge MS and Shannon R, 1983) was used 0.025 M $CaCl_2$ to activate prothrombin to thrombin, and thrombin would convert fibrinogen to fibrin. One hundred microliters of plasma or all of the fibrinogen solutions from precipitation was added into the test tube that was preincubated in the 37 °C waterbath. One hundred microliters of 0.025 M $CaCl_2$ was added into the test tubes. The clot was formed and incubated in the 37 °C waterbath for 30 minutes. Three milliliters of 5 M urea solution was added. They were stood at room temperature for 30 minutes. If factor XIII in plasma or all of the fibrinogen solutions was less than 2%, the clot was dissolved within 30 minutes. If the clot was not dissolved, the clot was stood at room temperature for 24 hours. The clot was observed every 6 hours.

The second method (Harmenning PD, 1987) was used 5 NIH units/ml thrombin to convert fibrinogen to fibrin. One hundred microliters of plasma or all of the fibrinogen solutions from precipitation was added into the test tube that was preincubated in the 37 °C waterbath. One hundred microliters of 5 NIH units/ml thrombin was added into the test tubes. The clot was formed and incubated in the 37 °C waterbath for 30 minutes. Three milliliters of 5 M urea solution was added. They were stood at room temperature for 30 minutes. If factor XIII in plasma or all of the fibrinogen solutions was less than 2%, the clot was dissolved within 30 minutes. If the clot was not dissolved, the clot was stood at room temperature for 24 hours. The clot was observed every 6 hours.

7. **Thrombin preparation**

Thrombin preparation in this study was based on *pI* and precipitation. According to its *pI*, prothrombin in plasma was precipitate when plasma was adjusted pH to 5.3, its *pI*. The conversion of prothrombin to thrombin was accelerated by $CaCl_2$, while thrombin was precipitated by precipitating agent.

The first method for thrombin preparation was used acetone as precipitating agent in the last step, an original method reported by Biggs R (1976). One hundred milliliters of CPD-plasma were diluted to 1,000 ml with distilled water. The pH of diluted plasma was adjusted to 5.3 with 2% acetic acid. It was centrifuged at 3,000 rpm for 10 minutes at 4 °C. The supernatant was discarded. The precipitate was dissolved in 25 ml of 0.85% NaCl. The pH of mixture was adjusted to 7.0 with 2% sodium carbonate (2% $Na_2CO_3$). Three milliliters of 0.25 M $CaCl_2$ was added and the coagulated fibrin was removed as it formed. It was stood at 4 °C for 2 hours for completely thrombin formation. The crude solution was purified by adding one volume of acetone to one volume of thrombin solution at room temperature. Thrombin was extracted from the precipitate with 25 ml of 0.85% NaCl. The saline extract was centrifuged at 3,000 rpm for 10 minutes at 4 °C. The supernatant was aliquot into 1 ml-vial for further study and stored at –20 °C.

The second method of thrombin preparation was modified from the first method by using absolute ethanol as precipitating agent. One hundred milliliters of CPD-plasma were diluted to 1,000 ml with distilled water. The pH of diluted plasma was adjusted to 5.3 with 2% acetic acid. It was centrifuged at 3,000 rpm for 10 minutes at 4 °C. The supernatant was discarded. The precipitate was dissolved in 25 ml of 0.85% NaCl. The pH of mixture was adjusted to 7.0 with 2% $Na_2CO_3$. Three milliliters of 0.25 M $CaCl_2$ was added and the coagulated fibrin was removed as it formed. It was stood at 4 °C for 2 hours for completely thrombin formation. The crude solution was

purified by adding one volume of absolute ethanol to one volume of thrombin solution at room temperature. Thrombin was extracted from the precipitate with 25 ml of 0.85% NaCl. The saline extract was centrifuged at 3,000 rpm for 10 minutes at 4 °C. The supernatant was aliquot into 1 ml-vial for further use and stored at –20 °C.

The third method of thrombin preparation was modified from the first method by using saturated ammonium sulfate as precipitating agent. One hundred milliliters of CPD-plasma were diluted to 1,000 ml with distilled water. The pH of diluted plasma was adjusted to 5.3 with 2% acetic acid. It was centrifuged at 3,000 rpm for 10 minutes at 4 °C. The supernatant was discarded. The precipitate was dissolved in 25 ml of 0.85% NaCl. The pH of mixture was adjusted to 7.0 with 2% sodium carbonate. Three milliliters of 0.25 M $CaCl_2$ was added and the coagulated fibrin was removed as it formed. It was stood at 4 °C for 2 hours for completely thrombin formation. The crude solution was purified by adding one volume of saturated ammonium sulfate to one volume of thrombin solution at room temperature. Thrombin was extracted from the precipitate with 25 ml of 0.85% NaCl. The saline extract was centrifuged at 3,000 rpm for 10 minutes at 4 °C. The supernatant was aliquot into 1 ml-vial for further use and stored at –20 °C.

The fourth method of thrombin preparation was modified from the first method by using polyethylene glycol as precipitating agent. One hundred milliliters of CPD-plasma were diluted to 1,000 ml with distilled water. The pH of diluted plasma was adjusted to 5.3 with 2% acetic acid. It was centrifuged at 3,000 rpm for 10 minutes at 4 °C. The supernatant was discarded. The precipitate was dissolved in 25 ml of 0.85% NaCl. The pH of mixture was adjusted to 7.0 with 2% sodium carbonate. Three milliliters of 0.25 M $CaCl_2$ was added and the coagulated fibrin was removed as it formed. It was stood at 4 °C for 2 hours for completely thrombin formation. The crude solution was purified by adding one volume of 50% of polyethylene glycol 8000 (50% PEG 8000) to one volume of thrombin solution at room temperature. Thrombin was extracted

from the precipitate with 25 ml of 0.85% NaCl. The saline extract was centrifuged at 3,000 rpm for 10 minutes at 4 °C. The supernatant was aliquot into 1 ml-vial for further use and stored at –20 °C.

The prepared thrombin and commercial thrombin (10 NIH units/ml) were diluted two fold. Its activity was determined by thrombin time. The desirable activity of thrombin by thrombin time method was not longer than 40 seconds.

## 8. Selection an appropriate method for fibrinogen and thrombin preparation

An appropriate method for fibrinogen preparation was selected by its fibrinogen concentration, quality of fibrinogen, and quantity and quality of factor XIII (FXIII). An appropriate method for thrombin preparation was selected by its thrombin-liked activity.

## 9. Fibrinogen and thrombin preparation in lyophilized form

Two milliliters of fibrinogen solution and thrombin solution were aliquot into 5-ml glass bottles. Fibrinogen was frozen at –20 °C overnight. Thrombin was divided into 2 groups. One was frozen at –20 °C overnight as slow-freeze thrombin. The other was frozen in the mixture of dry ice and absolute ethanol, and freeze at –20 °C overnight as quick-freeze thrombin. One layer of gauze was covered on the bottle without its cap. They were put in the Labconco Freeze Dryer III that already preset its pressure and temperature. They were lyophilized overnight. The lyophilized fibrinogen and the lyophilized thrombin were stored at –20 °C for further determinations.

**PART II**

1. **Composition of prepared fibrin glue**

ε-aminocaproic acid (EACA) was used as antifibrinolytic agent for fibrin glue that prepared by this present study. Three concentration of EACA (7.5, 10.0 and 12.5 mg/ml) were added to fibrinogen from repeat cryoprecipitation and cryoprecipitation to define the least concentration of EACA for preventing autofibrinolysis of fibrin glue.

The first set of prepared fibrin glue was as followed. The thrombin solution of prepared fibrin glue was consisted of 2 mg/ml of gentamicin in 1 ml of 40 mmol/L $CaCl_2$ and lyophilized quick-freeze or slow-freeze thrombin. The fibrinogen solution of prepared fibrin glue was consisted of 75 µl of 100 mg/ml EACA, 925 µl of distilled water and lyophilized fibrinogen from repeat cryoprecipitation or cryoprecipitation. The final concentration of EACA in this fibrinogen solution was 7.5 mg/ml.

The second set of prepared fibrin glue was as follows. The thrombin solution of prepared fibrin glue was consisted of 2 mg/ml of gentamicin in 1 ml of 40 mmol/L $CaCl_2$ and lyophilized quick-freeze or slow-freeze thrombin. The fibrinogen solution of prepared fibrin glue was consisted of 100 µl of 100 mg/ml EACA, 900 µl of distilled water and lyophilized fibrinogen from repeat cryoprecipitation or cryoprecipitation. The final concentration of EACA in this fibrinogen solution was 10.0 mg/ml.

The third method of prepared fibrin glue was as followed. The thrombin solution of prepared fibrin glue was consisted of 2 mg/ml of gentamicin in 1 ml of 40 mmol/L $CaCl_2$ and lyophilized quick-freeze or slow-freeze thrombin. The fibrinogen solution of prepared fibrin glue was consisted of 125 µl of 100 mg/ml

EACA, 825 µl of distilled water and lyophilized fibrinogen from repeat cryoprecipitation or cryoprecipitation. The final concentration of EACA in this fibrinogen solution was 12.5 mg/ml.

All of these sets of prepared fibrin glue were performed in the test of solubility, stability, adhesive strength and elasticity.

2. **Determination of the stability of fibrin glue**

Determination of the stability of fibrin glue was modified from the method reported by Chuansumrit, *et al.* (1997). There were two ratio of the two solution of prepared fibrin glue in these studies.

First ratio was contained 1:1 of fibrinogen and thrombin solution. One hundred microliters of fibrinogen solution and thrombin solution were added into 12x75 mm test tube in the 37 °C waterbath. The clot was formed. The clot was observed every 6 hours until the clot was completely dissolved.

Second ratio was contained 2:1 of fibrinogen and thrombin solution. Sixty-six microliters of fibrinogen solution and 33 µl of thrombin solution were added into 12x75 mm test tube in the 37 °C waterbath. The clot was formed. The clot was observed every 6 hours until the clot was completely dissolved.

Fibrin glue prepared by Thai Red Cross Society was also determined its stability. The test was performed in 1:1 of fibrinogen and thrombin solution, the ratio that recommended by Thai Red Cross Society. One hundred microliters of fibrinogen solution and thrombin solution were added into 12x75 mm test tube in the 37 °C waterbath. The clot was formed. The clot was observed every 6 hours until the clot was completely dissolved.

3. **Determination of adhesive strength of fibrin glue**

MC-1 apparatus was modified from Seidentop KH, *et al.* (1988) and Park JJ, *et al.* (1997) for measuring the adhesive strength of fibrin glue. It was shown in figure 12. It was constructed from acrylic plate, which is composed of three parts. The first part is called "STAND" that used for hold the "TOP" by the holder while measuring the adhesive strength (fig. 13). The second part is called "TOP" that used for applied the fibrin glue (fig. 14). It is composed of a 90x40 mm acrylic plate, a 20x20 mm acrylic plate, and a 12x12 mm acrylic plate that was cut to the 10x10 mm inner area. The height of 12x12 mm of acrylic plate is 1 mm. So the volume within the block of the "TOP" is 100 $mm^3$ (approximately 100 μl). The third part is called "BOTTOM" that used for covering the "TOP" after the fibrin glue was applied (fig. 15). This is a 55x40 mm acrylic plate that had a string for hanging the S-shaped wire (fig. 16). After applying the fibrin glue in the block of the "TOP", the "BOTTOM" was immediately covered on it. After 10 minutes or 30 minutes, this test set was placed on the holder of the "STAND". The S-shaped wire was placed on the string under the "BOTTOM" for adding a weight. The weight that used for measuring the adhesive strength was connected to a loop string (fig. 16). Two ratios of the two solutions of prepared fibrin glue in the present study were used in this study.

First ratio was contained 1:1 of fibrinogen and thrombin solution. Fifty microliters of fibrinogen solution prepared in the present study was applied into the 1x1 cm block on the acrylic plate "TOP". Fifty microliters of thrombin solution prepared in this study was applied into the block respectively. The acrylic plate "BOTTOM" was covered on the block immediately. The timer was started immediately when the thrombin solution was applied. After 10 and 30 minutes, the test set was placed on the STAND of MC-1 apparatus. One hundred grams weight was added to the S-shape wire that placed on the string under the BOTTOM. Ten grams weight was added every 30 seconds

until the BOTTOM was separated from the TOP. The total weight and the times were recorded.

Second ratio was contained 2:1 of fibrinogen and thrombin solution. Sixty six microliters of fibrinogen solution prepared in the present study was applied into the 1x1 cm block on the acrylic plate "TOP" and 33 μl of thrombin solution prepared in the present study was applied into the block respectively. The acrylic plate "BOTTOM" was covered on the block immediately. The timer was started immediately when the thrombin solution was applied. After 10 and 30 minutes, the test set was placed on the stand of MC-1 apparatus. One hundred grams weight was added to the S-shape wire that placed on the string under the BOTTOM. Ten grams weight was added every 30 seconds until the BOTTOM was separated from the TOP. The total weight was recorded.

Fibrin glue prepared by Thai Red Cross Society was also determined its adhesive strength. The test was performed in 1:1 of fibrinogen and thrombin solution, the ratio that recommended by Thai Red Cross Society. One hundred microliters of fibrinogen solution was applied into the 1x1 cm block on the acrylic plate "TOP" and 100 μl of thrombin solution was applied into the block respectively. The acrylic plate "BOTTOM" was covered on the block immediately. The timer was started immediately when the thrombin solution was applied. After 10 and 30 minutes, the test set was placed on the stand of MC-1 apparatus. One hundred grams weight was added to the S-shape wire that placed on the string under the BOTTOM. Ten grams weight was added every 30 seconds until the BOTTOM was separated from the TOP. The total weight was recorded. Each was replicated for 5 times.


**Figure 12** MC-1 Apparatus


**Figure 13** STAND of MC-1 apparatus


**Figure 14** TOP of MC-1 apparatus

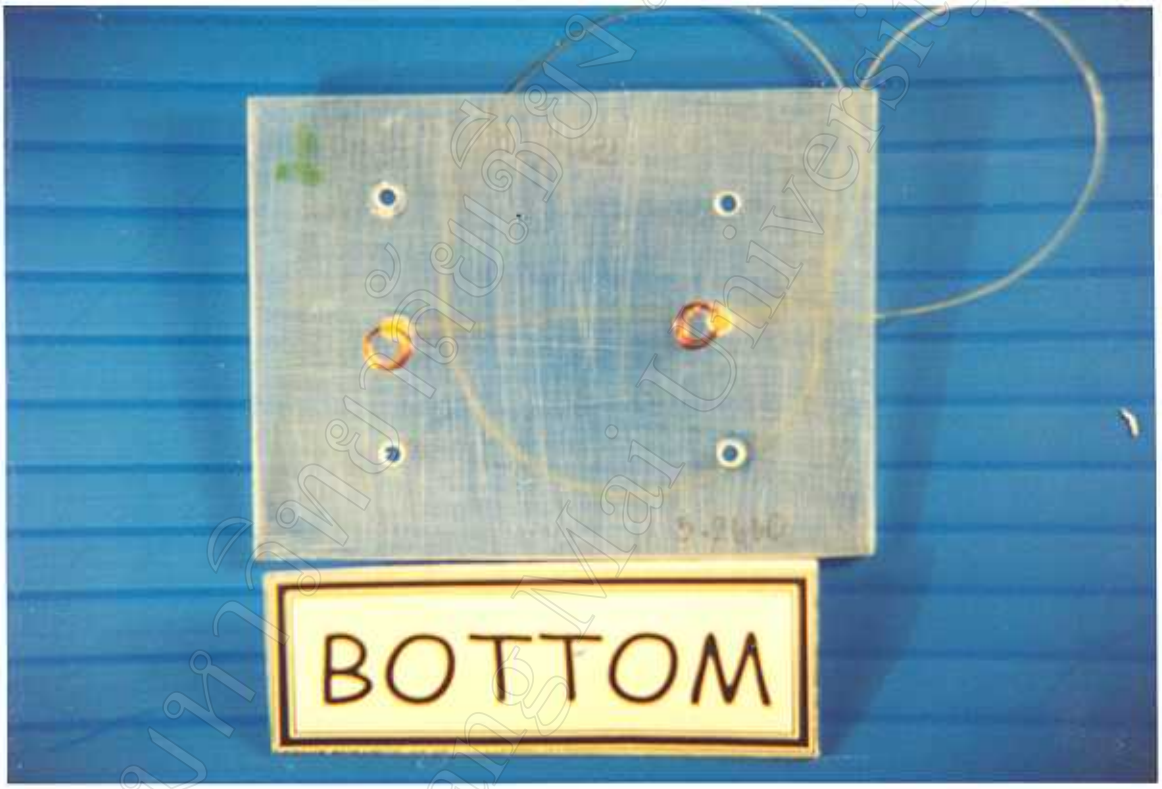


**Figure 15** BOTTOM of MC-1 apparatus


**Figure 16** S-shaped wire (left) and weights (right) of MC-1 apparatus

4. **Determination of the elasticity of fibrin glue**

Determination of the elasticity of prepared fibrin glue was modified from Burnouf-Rodosevich M, *et al.* (1990). There were two ratios of the two solutions of 3 sets of prepared fibrin glue in these studies.

First ratio was contained 1:1 fibrinogen and thrombin solution. Fifty microliters of fibrinogen solution was applied into the 1x1 cm block on the acrylic plate "TOP" and 50 µl of thrombin solution was applied into the block respectively. The timer was immediately started when the thrombin solution was applied. After 30 minutes, the fibrin clot on the block was softly pulled with a forceps. The height of the fibrin film was recorded in millimeters. Each was preformed in duplicate.

Second ratio was contained 2:1 fibrinogen and thrombin solution. Sixty six microliters of fibrinogen solution was applied into the 1x1 cm block on the acrylic plate "TOP" and 33 µl of thrombin solution was applied into the block respectively. The timer was immediately started when the thrombin solution was applied. After 30 minutes, the fibrin clot on the block was softly pulled with a forceps. The height of the fibrin film was recorded in millimeters. Each was performed in duplicate.

Fibrin glue prepared by Thai Red Cross Society was also determined its elasticity. The test was performed in ratio was contained 1:1 fibrinogen and thrombin solution, the ratio that recommended by Thai Red Cross Society. One hundred microliters of fibrinogen solution was applied into the 1x1 cm block on the acrylic plate "TOP" and 100 µl of thrombin solution was applied into the block respectively. The timer was started immediately when the thrombin solution was applied. After 30 minutes, the fibrin clot on the block was softly pulled with a forceps. The height of the fibrin film was recorded in millimeters. It was performed in duplicate.

5. **Comparisons of fibrin glue of the present study with Thai Red Cross Society preparation**

Fibrinogen concentration determined by modified thrombin time method and Ratnoff's method in fibrinogen solution from cryoprecipitation and repeat cryoprecipitation in non-lyophilized and lyophilized form by the present study, and cryoprecipitate prepared by Thai Red Cross Society were compared. The stability, the adhesive strength and the elasticity of fibrin glue prepared by the present study, and prepared by Thai Red Cross Society were compared.