Original Article
Diagnostic Hematology

Ann Lab Med 2025;45:484-492
https://doi.org/10.3343/alm.2024.0688
ISSN 2234-3806 • eISSN 2234-3814

ANNALS OF LABORATORY MEDICINE

# A Novel Fibrinogen Assay Using Recombinant Batroxobin and Carboxymethyl Chitosan: Carboxymethyl Chitosan Stimulates the Enzymatic Activity of Recombinant Batroxobin

Jung-Ah Kim, M.D., Ph.D.[1], Eunhye Ko, M.S.[2], Yongje Woo, Ph.D.[2], Young-Doug Sohn, Ph.D.[2], Jong-Tak Kim, Ph.D.[2], Jaewoo Song, M.D., Ph.D.[3], and Rojin Park, M.D., Ph.D.[1]

[1]Department of Laboratory Medicine, Soonchunhyang University Seoul Hospital, Seoul, Korea; [2]NCbit R&D Center, NCbit, Inc., Seongnam, Korea; [3]Department of Laboratory Medicine, Yonsei University College of Medicine, Seoul, Korea

**Background:** The Clauss assay is widely used to quantify blood fibrinogen levels in clinical laboratories. However, by relying on thrombin as the main reagent, the Clauss assay is susceptible to interference from thrombin inhibitors, such as heparin or direct thrombin inhibitors. Here, we developed an innovative fibrinogen assay utilizing both recombinant batroxobin (rBat) and carboxymethyl chitosan (CMCS).

**Methods:** Various biopolymers were tested to identify a suitable candidate that could enhance rBat-induced fibrin clot formation. Chromogenic substrate hydrolysis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis showed that CMCS potentiated rBat activity. Consequently, we formulated a novel fibrinogen assay reagent, ANYFIB.C, comprising rBat and CMCS. We compared ANYFIB.C fibrinogen with an established reagent (HemosIL fibrinogen-C) with 96 clinical samples using an ACL-TOP 700 coagulation analyzer. We also evaluated the interfering effects of thrombin inhibitors on fibrinogen measurements.

**Results:** CMCS significantly enhanced the enzymatic activity of rBat and dose-dependently reduced plasma clotting times. ANYFIB.C fibrinogen levels were comparable with those of HemosIL fibrinogen-C, with the 95% confidence intervals of the Passing–Bablok regression intercept and slope being −7.4797 to 6.0185 and 0.9581 to 1.0116, respectively. No significant interference was observed with heparin concentrations up to 10 U/mL or dabigatran concentrations up to 600 µg/L in the ANYFIB.C fibrinogen assays. In contrast, the HemosIL fibrinogen-C reagent demonstrated inhibitory interference at dabigatran concentrations as low as 150 µg/L.

**Conclusions:** Our results suggest that ANYFIB.C (a mixture of CMCS and rBat) can be used to measure blood fibrinogen levels effectively and protect from thrombin inhibitor interference.

**Key Words:** ANYFIB.C fibrinogen assay reagent, Carboxymethyl chitosan, Clauss fibrinogen assay, Dabigatran, Heparin, Recombinant batroxobin

**Received:** December 22, 2024
**Revision received:** February 5, 2025
**Accepted:** May 6, 2025
**Published online:** June 12, 2025

**Corresponding author:**
Rojin Park, M.D., Ph.D.
Department of Laboratory Medicine,
Soonchunhyang University Seoul Hospital,
59 Daesagwan-ro, Yongsan-gu,
Seoul 04401, Korea
E-mail: rpark@schmc.ac.kr

**Co-corresponding author:**
Jaewoo Song, M.D., Ph.D.
Department of Laboratory Medicine,
Yonsei University College of Medicine,
50-1 Yonsei-ro, Seodaemun-gu,
Seoul 03722, Korea
E-mail: labdx@yuhs.ac

## INTRODUCTION

Fibrinogen, a 340-kDa plasma protein produced in the liver [1], plays a critical role in hemostasis by forming fibrin clots during blood coagulation upon activation by thrombin [2]. Normal plasma fibrinogen levels range from 2 to 4 g/L, with elevated levels observed in acute infections, inflammatory diseases, malignancies, and cardiovascular conditions [3]. Conversely, fibrinogen levels decrease in disseminated intravascular coagulation, end-stage liver disease, and inherited fibrinogen deficiencies, including afibrinogenemia, hypofibrinogenemia, and dysfibrinogenemia [4].

Blood fibrinogen levels can be measured using antigen- and activity-based methods [5]. Similarly, fibrinogenemia and hypofibrinogenemia (characterized by decreased fibrinogen concentrations) can be identified through antigen- and activity-based assays, whereas dysfibrinogenemia can only be detected in activity-based assays. Consequently, fibrinogen activity assays are employed as the primary diagnostic method in clinical laboratories, and antigen assays provide additional utility in differentiating between fibrinogen deficiency and dysfunction. Afibrinogenemia and hypofibrinogenemia are typically associated with an increased risk of bleeding. In contrast, dysfibrinogenemia may manifest clinically with either bleeding or thrombotic complications [6]. The Clauss assay is the standard method for measuring fibrinogen activity [7]. However, because it utilizes thrombin as the primary reagent, the assay results can be affected by high concentrations of thrombin inhibitors, such as heparin and direct thrombin inhibitors (DTIs) [8].

Thrombin is an inherently unstable enzyme [9], and its extraction from human or animal plasma introduces challenges in standardization and potential risks for infectious diseases [10, 11]. To overcome these limitations, batroxobin, a thrombin-like serine protease derived from the venom of *Bothrops jararaca* or *Bothrops atrox*, can serve as an alternative to thrombin in fibrinogen assays [12, 13]. Unlike thrombin, which cleaves both the Aα and Bβ chains of fibrinogen to release fibrinopeptides A and B and activates factor XIII (FXIII) to form cross-linked des-AB fibrin clots, batroxobin only cleaves the Aα chain, releasing fibrinopeptide A and forming non-cross-linked des-AA fibrin clots [14]. Batroxobin exhibits greater stability and is not inhibited by heparin or antithrombin, making it potentially advantageous for fibrinogen measurements [15]. However, batroxobin can induce fibrin clots at low fibrinogen levels (<50 mg/dL) that are not easily detected with optical methods. We overcame this optical detection issue by developing a novel fibrinogen reagent, ANYFIB.C, a combination of recombinant batroxobin (rBat) and carboxymethyl chitosan (CMCS). The ANYFIB.C reagent facilitated polymer scaffold formation, thereby enhancing clot detectability.

In this study, we assessed the comparability of fibrinogen assay results obtained using ANYFIB.C and HemosIL fibrinogen-C (a widely used reagent). Additionally, we examined whether ANYFIB.C could counteract the interfering effects of thrombin inhibitors.

## MATERIALS AND METHODS

This study was conducted in compliance with ethical guidelines set by the Institutional Review Board (IRB) of Soonchunhyang University Seoul Hospital, Seoul, Korea (IRB approval number SCHUH 2020-09-017). The requirement for written informed consent was waived by the IRB.

### Reagents

Natural batroxobin (nBat) was purchased from DSM (Pentapharm, Basel, Switzerland). Human α-thrombin, porcine skin gelatin, human fibrinogen, and bovine fibrinogen were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Carboxymethyl cellulose (CMCel) was purchased from Duksan (Ansan, Korea). Hyaluronic acid (HA) was purchased from SK Bioland (Cheonan, Korea). Dextran 40 was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Human plasma was purchased from Innovative Research, Inc. (Southfield, MI, USA). Porcine plasma was purchased from CRONEX (Seoul, Korea). A chromogenic substrate for thrombin, H-D-Phe-Pip-Arg-*p*-nitroanilide (BIOPHEN CS–01[38]), was purchased from Hyphen BioMed (Paris, France). HemosIL fibrinogen-C was purchased from Instrumentation Laboratory (Bedford, MA, USA). We expressed and purified rBat and used it after confirming its activity, as previously described [16]. CMCS was purchased from HMC+ (Halle, Germany).

### Biopolymers, fibrinogen preparations, and clotting assays: screening for an optimal biopolymer displaying synergistic activity with rBat

Biopolymers of CMCS, CMCel, dextran 40, HA, and gelatin were each dissolved in 10 mM Tris-HCl buffer (pH 6.8) solution at 2.0% (w/v), and the resulting gelatin solution was incubated at 37°C using a water bath. Mixtures of rBat with or without biopolymers were prepared in 10 mM Tris-HCl (pH 6.8). The final concentrations of rBat and the biopolymers expressed as batroxobin unit were 5.00 BU/mL and 1.0% (w/v), respectively.

To determine the plasma clotting time, 0.3 mL human plasma (Innovative Research, Inc., Novi, MI, USA) was thawed rapidly in a 37°C water bath and incubated at 37°C for 3 min, after which 0.1 mL of each rBat mixture was added. The plasma clotting time was measured using a thrombotimer (Behnk Elektronik, Germany). Fibrinogen clotting assays were performed with an ACL 10000 coagulation analyzer (Instrumentation Laboratory, Bedford, MA, USA). Human fibrinogen (100 µL, 2 mg/mL) was incubated at 37°C in the instrument for 60 sec, and then 50 µL of rBat or one of the rBat mixtures was added. The time to clotting after adding each mixture was detected by measuring the absorbance at 405 nm. Various concentrations of CMCS (0, 2.5, 5.0, 7.5, 10.0 mg/mL) in the rBat mixtures were tested to investigate the dose–response relationship of CMCS in terms of the clotting time. Subsequently, plasma and fibrinogen clotting times were measured, as described above. All testing was performed in triplicate.

### Effect of CMCS on rBat–, nBat–, or thrombin–catalyzed clotting times

We used rBat and nBat from *Bothrops moojeni* (Pentapharm) as thrombin-like enzymes and human thrombin (Sigma-Aldrich) when measuring plasma and fibrinogen clotting times. Mixtures containing each enzyme and CMCS were prepared, resulting in final CMCS, rBat, nBat, and thrombin concentrations of 1.0% (w/v), 5.00, 5.00 BU/mL, and 1.00 NIH unit/mL in 10 mM Tris-HCl buffer (pH 6.8), respectively. Plasma and fibrinogen clotting times were measured as described above. Reactions were initiated by adding rBat, nBat, or thrombin to the plasma or fibrinogen solutions, with or without CMCS.

### Effect of CMCS on rBat enzymatic activity

To investigate the effect of CMCS on the enzyme activity of rBat, we conducted the following two experiments.

### Measuring chromogenic substrate hydrolysis by rBat (with or without CMCS) and related kinetic parameters

CMCS (final concentration, 0.1% w/v) was mixed with the thrombin chromogenic substrate, BIOPHEN CS–01(38), i.e., H-D-Phe-Pip-Arg-*p*-nitroanilide (Hyphen BioMed), and incubated with rBat at 37°C in 10 mM Tris-HCl buffer (pH 6.8) [16]. Each 200 µL reaction included 160 µL buffer, 1 mM substrate (20 µL), and rBat (5.00 BU/mL, 20 µL), with or without CMCS (1.0% w/v). CMCS was not included in control reactions. Substrate hydrolysis was monitored by measuring the increase in absorbance at 405 nm for 11 min at 1 min intervals. Seven substrate concentrations (0, 25, 50, 100, 200, 300, and 400 µM) were used to determine the maximum velocity ($V_{max.}$), Michaelis–Menton constant ($K_m$), and catalytic constant ($k_{cat}$) of rBat. The $V_{max.}$, $K_m$, and $k_{cat}$ were calculated using the Lineweaver–Burk equation. All assays were performed three times, and the absorbances at 405 nm were measured using a TECAN microplate reader (Tecan Austria GmbH, Grödig, Austria).

### Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of rBat–induced Des–A fibrin clotting, with or without CMCS (fibrinopeptide A–release assay)

Human fibrinogen was adjusted to 2 mg/mL in solution. After adding rBat (final concentration, 1.25 BU/mL), the solution was incubated with or without CMCS (final concentration, 0.125% w/v) for 0, 10, 20, or 30 sec. Each reaction was terminated immediately by adding 0.5 mM Tris-HCl buffer (pH 8.0) containing 20% glycerol (v/v), 4% SDS (v/v), 0.05% bromophenol blue (w/v), and 10% β-mercaptoethanol (v/v), followed by boiling for 5 min. The digested α-chains of fibrinogen were analyzed via 10% SDS-PAGE.

### Effects of fibrinogen and the ANYFIB.C fibrinogen reagent on clot formation activity at low concentrations

Fibrinogen clotting times were measured on an ACL 10000 coagulation analyzer (Instrumentation Laboratory). Saline solutions were prepared with human fibrinogen at various concentrations (30, 40, 50, 75, 100, 50, and 200 mg/dL). Subsequently, 100 µL of each human fibrinogen sample was incubated at 37°C in the instrument for 60 sec, after which a 50 µL mixture containing thrombin (1.00 NIH U/mL), rBat (final 5.00 BU/mL), or rBat/CMCS (final 1.0% w/v) was added. The time until clot formation after adding the enzyme was detected by measuring the absorbance at 405 nm.

### Preparing the ANYFIB.C fibrinogen assay reagent

Based on the results shown in Table 1, we developed the ANYFIB.C fibrinogen reagent composed of a mixture of rBat and CMCS. HemosIL calibration plasma was used to calibrate both the HemosIL fibrinogen-C and ANYFIB.C reagents to measure fibrinogen activity with an ACL-TOP 700 coagulation analyzer.

### Assaying the effects of heparin or dabigatran on fibrinogen levels

Plasma samples with normal (210 and 270 mg/dL) or abnormal (470 mg/dL) fibrinogen levels were spiked with heparin (0, 0.5,


https://doi.org/10.3343/alm.2024.0688

**Table 1.** Fibrinogen clotting times measured with thrombin, rBat, or the rBat–CMCS mixture

| Fibrinogen (mg/dL) | Thrombin (1.00 NIH U/mL) | rBat (5.00 BU/mL) | rBat+CMCS |
|---|---|---|---|
| | Fibrinogen clotting time (sec)* | | |
| 200 | 26.83±0.47 | 24.97±0.51 | 16.37±0.15† |
| 150 | 32.30±4.18 | 26.50±0.50 | 17.23±0.25† |
| 100 | 38.13±0.67 | 25.93±0.45 | 19.13±0.31† |
| 75 | 42.97±0.45 | 32.67±1.93 | 27.53±1.33† |
| 50 | 73.70±2.91 | 74.13±6.33 | 48.13±0.64† |
| 40 | 84.03±2.65 | ND | 70.13±1.06† |
| 30 | ND | ND | 88.76±0.60 |

*The data shown represent the mean±SD.
†Significant differences ($P<0.05$) were determined via Kruskal-Wallis test.
Abbreviations: rBat, recombinant batroxobin; CMCS, carboxymethyl chitosan; NIH, National Institutes of Health; ND not detected.

1, 5, or 10 U/mL) or dabigatran (0, 150, 300, or 600 mg/L) to investigate their effects on fibrinogen levels. The fibrinogen levels were measured in an ACL-TOP700 coagulation analyzer.

### Comparability between the ANYFIB.C and HemosIL fibrinogen-C reagents

We prepared 96 residual plasma samples by centrifuging whole blood collected from hospitalized patients in tubes containing 3.2% sodium citrate (Becton, Dickinson, Franklin Lakes, NJ, USA). Plasma fibrinogen levels were measured in the clinical samples after spiking them with various concentrations of the ANYFIB.C or HemosIL fibrinogen-C reagent, and the results were compared according to the CLSI H57-A guidelines [17].

### Statistical analysis

All statistical analyses were performed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA) and MedCalc Statistical Software, version 14.12.0 (MedCalc Software, Mariakerke, Belgium). Differences in the clotting times observed with thrombin, rBat, or the rBat–CMCS mixture were detected using Kruskal-Wallis test. Differences in clotting times (with or without CMCS) were tested using the Mann–Whitney U test. The comparability analysis between the HemosIL fibrinogen-C and ANYFIB.C reagents was based on Passing–Bablok regression analysis, Spearman's correlation coefficient, and a Bland–Altman difference plot.

## RESULTS

### Screening for an optimal biopolymer that synergized with rBat to shorten clotting times

First, we compared the effects of various biopolymers on rBat-induced plasma and fibrinogen clotting times (Fig. 1A and 1B). Among those biopolymers, the CMCS–rBat mixture significantly shortened plasma and fibrinogen clotting times more than the others. In addition, CMCS dose-dependently shortened the rBat-induced plasma (Fig. 1C) and fibrinogen (Fig. 1D) clotting times.

### Effect of CMCS on the enzyme activities of rBat, nBat, or thrombin

We also investigated the synergistic effects of CMCS with nBat and thrombin. During plasma clotting (Fig. 2A), nBat and thrombin showed similar synergism with CMCS to that observed with rBat, although rBat showed a slightly better effect on fibrinogen clotting (Fig. 2B). Similar results were obtained for other thrombin-like enzymes, such as ancrod (Malayan pit viper) and botropase (*Bothrops jararaca*) (data not shown).

### CMCS increased the enzymatic activity of rBat

To understand how CMCS further reduced the rBat-catalyzed clotting time, we studied the effect of CMCS on the enzymatic activity of rBat. Michaelis–Menten kinetics were applied to show the absorbance values induced by rBat (final concentration, 0.50 BU/mL) with or without CMCS (final concentration, 0.1% w/v). The absorbance values were measured for 11 min, using the chromogenic substrate BIOPHEN CS–01(38) for thrombin, at various concentrations ranging from 0 to 400 μM. BIOPHEN CS–01(38) is widely used to measure the enzymatic activity of thrombin-like enzymes [16]. The initial rates versus the thrombin BIOPHEN CS–01(38) concentration were hyperbolic (Fig. 3A), and these plots were used to derive the apparent $K_m$, $k_{cat}$, and maximal velocity ($V_{max}$) of the reactions (Fig. 3A).

The $V_{max}$ increased from 168.1 to 303.1 absorbance units/min in the presence of CMCS, and the $k_{cat}$ increased from 3.7 to 6.7/min, compared with the control values obtained in the absence of CMCS. The $K_m$ values were slightly higher in the presence of CMCS than in the absence of CMCS. These data imply that CMCS increased the enzymatic activity of rBat by increasing its catalytic efficacy rather than promoting enzyme–substrate binding. To directly demonstrate that CMCS increased the enzymatic activity of rBat, we performed an SDS-PAGE analysis of bovine fibrinogen treated with rBat in the presence or absence of CMCS. Fig. 3B shows that fibrinopeptide A was released faster in the

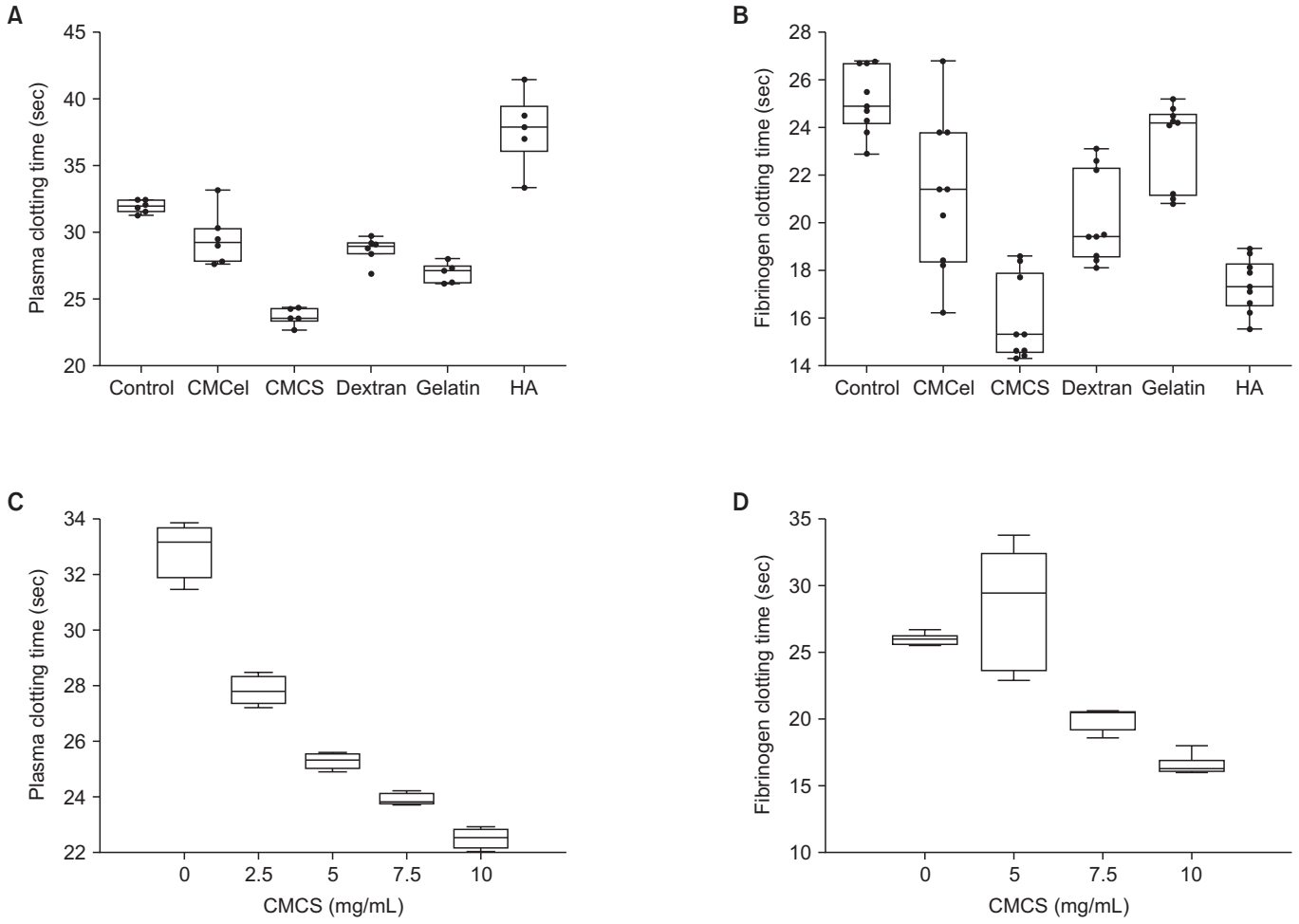



**Fig. 1.** Screening for a biopolymer exhibiting synergistic activity with rBat in des-AA fibrin clot formation. (A, B) The addition of various biopolymers enhanced rBat-induced clotting in plasma (A) and fibrinogen (B). Reactions were initiated by adding rBat (final 5.00 BU/mL) to plasma or fibrinogen solutions containing biopolymers (final 1.0% w/v). (C, D) CMCS shortened rBat-triggered plasma (C) and fibrinogen (D) clotting times in a dose-dependent manner. The data represent the mean values of triplicate measurements.
Abbreviations: CMCS, carboxymethyl chitosan; CMCel, carboxymethyl cellulose; HA, hyaluronic acid.

presence of CMCS than in its absence. That is, fibrinogen Aα-chain cleavage to form Des-A fibrin took 20 or 30 sec in the presence or absence of CMCS, respectively, indicating that CMCS stimulated the enzymatic activity of rBat for both the chromogenic substrate and fibrinogen.

## Using an rBat–CMCS mixture to measure functional fibrinogen levels

CMCS enhanced the enzymatic activity of rBat and the strength of rBat-induced clotting (Fig. 3 and Supplemental Data Fig. S1). Based on the CMCS characteristics, we developed a mixture of rBat and CMCS as a fibrinogen assay reagent. We determined the minimal fibrinogen concentration over which the rBat-induced fibrinogen clotting time could be measured. Human fibrinogen was progressively diluted with saline, and the clotting times of the diluted fibrinogen samples were measured using an ACL 10000 instrument. Table 1 shows that the fibrinogen clotting time could be measured with 50 mg/dL fibrinogen without CMCS or with 30 mg/dL of fibrinogen plus CMCS. The fibrinogen clotting time was significantly shorter with CMCS than with rBat alone or rBat plus thrombin (1.00 NIH U/mL). These results indicate that the rBat–CMCS could be used as a diagnostic reagent for fibrinogen assays.

## Comparability of ANYFIB.C with the HemosIL fibrinogen-C reagent

We used 96 clinical samples with fibrinogen concentrations ranging from 58 to 1,040 mg/dL (based on calibration with He-


https://doi.org/10.3343/alm.2024.0688

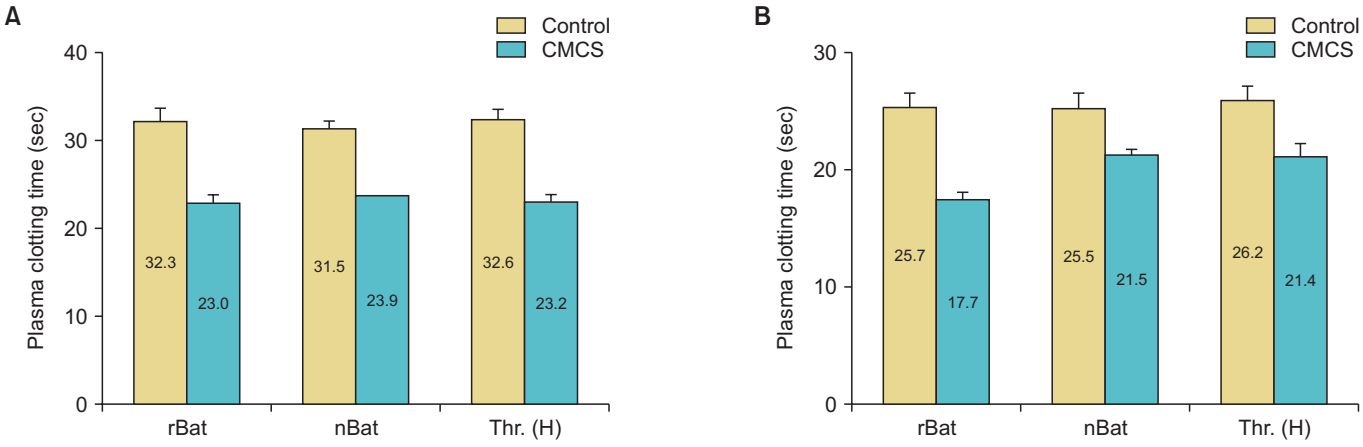
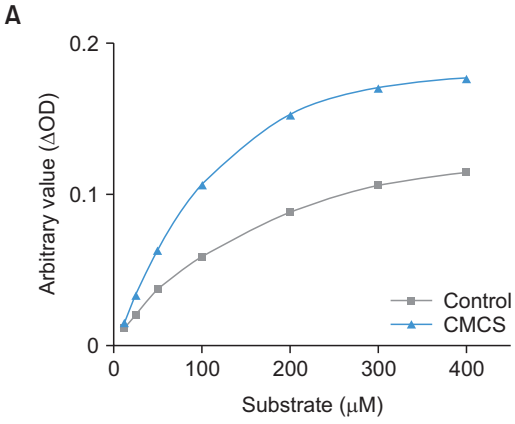



**Fig. 2.** Effects of CMCS on the enzyme activity of rBat, nBat, and human thrombin. (A, B) CMCS exerts a synergistic effect with rBat, nBat, and human thrombin on plasma (A) and fibrinogen (B) clotting times. The clotting times following treatment with rBat, nBat, or human thrombin plus CMCS were significantly shorter than those observed without CMCS, as assessed using the Mann–Whitney U test (N = 3, $P < 0.05$). Reactions were initiated by adding each enzyme to plasma or fibrinogen with or without CMCS. Data represent the mean of triplicate measurements.
Abbreviations: CMCS, carboxymethyl chitosan; rBat, recombinant batroxobin; nBat, natural batroxobin; Thr. (H), human thrombin.




| | Control | CMCS |
|---|---|---|
| $V_{max}$ ($10^{-3}$, ΔOD·$min^{-1}$) | 168.1±2.9 | 303.1±18.0* |
| $K_m$ (mM) | 0.181±0.004 | 0.200±0.018* |
| $k_{cat}$ ($10^{-3}$, $min^{-1}$) | 3.70±0.07 | 6.70±0.40* |
| $k_{cat}/K_m$ ($10^{-3}$, $min^{-1}$·$mM^{-1}$) | 20.6±0.49 | 33.8±1.04* |

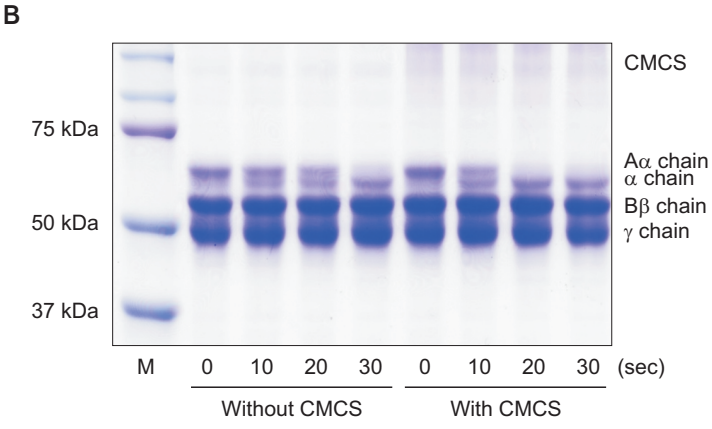



**Fig. 3.** Analysis of the enzyme activity of rBat by chromogenic substrate hydrolysis (A) and SDS-PAGE of rBat-induced fibrin clot (B). (A, B) The chromogenic substrate BIOPHEN CS-01(38) (A) and fibrinogen (B) were used as substrates. The data shown in (A) represent the mean ± standard deviation.
*Statistically significant differences were based on comparisons with values obtained with rBat, as determined using the Mann–Whitney U test (N = 3, $P < 0.05$).
Abbreviations: OD, optical density unit; CMCS, carboxymethyl chitosan; $V_{max}$, maximum velocity; $K_m$, Michaelis–Menton constant; $k_{cat}$, catalytic constant.

mosIL fibrinogen-C) for compatibility testing. Passing–Bablok regression analysis showed that the 95% confidence intervals for the intercept and slope were −7.4797 to 6.0185 and 0.9581 to 1.0116, respectively, indicating that both reagents were comparable within the studied concentration range (Fig. 4).

### Effects of heparin and dabigatran on the fibrinogen assay

No significant inhibition of fibrinogen levels was detected with

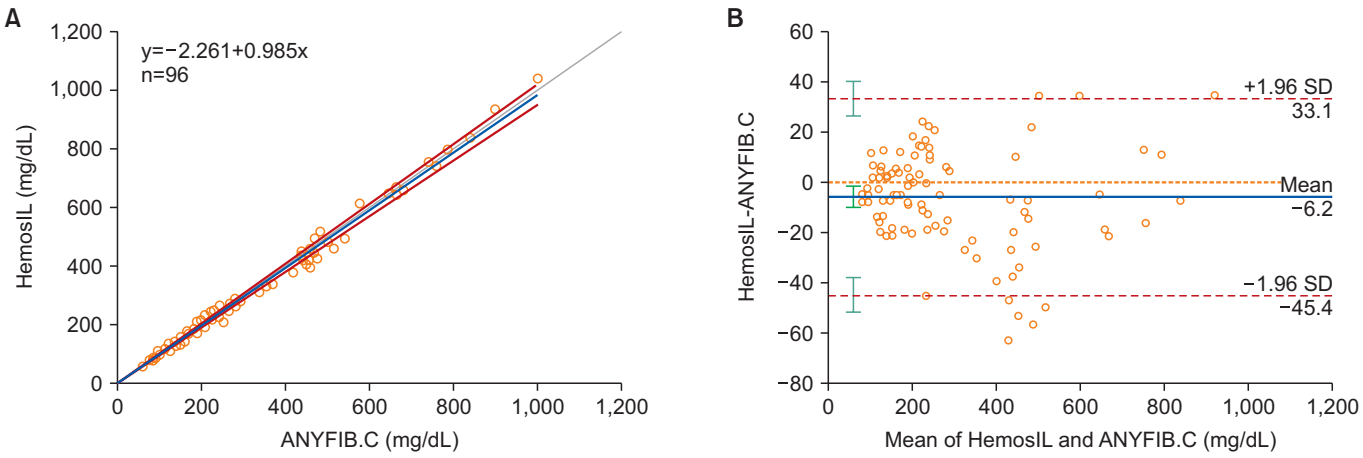



**Fig. 4.** Comparison of ANYFIB.C with HemosIL fibrinogen-C. Ninety-six clinical samples covering a low to high fibrinogen concentration range (58–1040 mg/dL with HemosIL fibrinogen-C) were used for the compatibility test. Passing-Bablok regression analysis (A) and Bland-Altman plot (B) show that the two reagents are comparable within the studied concentration range. The 95% confidence intervals for intercept and slope are −7.480 to 6.019 and 0.958 to 1.012, respectively.

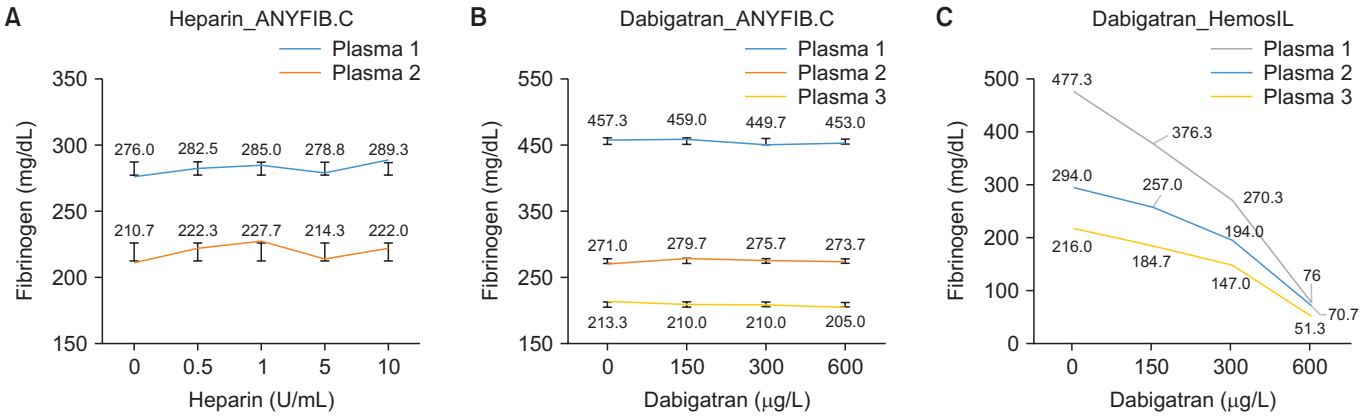



**Fig. 5.** Effects of heparin and dabigatran on fibrinogen levels measured using the ANYFIB.C and HemosIL fibrinogen reagents. (A–C) The ANYFIB.C reagent was not influenced by increasing concentrations of heparin (A) and dabigatran (B), a direct thrombin inhibitor, when compared with the HemosIL fibrinogen-C reagent (C). The data shown represent the mean value of triplicate measurements.

heparin (up to 10 U/mL) in two plasma samples containing the ANYFIB.C reagent in the ACL-TOP 700 coagulation analyzer (Fig. 5A). ANYFIB.C showed no inhibition even in the presence of up to 600 mg/L dabigatran (Fig. 5B), whereas the HemosIL fibrinogen-C reagent began showing inhibitory effects with 150 mg/L dabigatran (Fig. 5C). In *in vitro* spiking experiments (Fig. 5C), the fibrinogen levels measured with the HemosIL fibrinogen-C reagent decreased by at least >10% at dabigatran concentrations above 150 mg/L and decreased by >75% at a concentration of 600 mg/L. These data indicate that the activity of the thrombin-like enzyme, batroxobin, was not affected by thrombin inhibitors such as heparin or dabigatran.

## DISCUSSION

Accurate measurement of blood fibrinogen levels across a wide range is crucial due to the clinical implications of elevated fibrinogen concentrations, which increase risks for cardiovascular diseases (such as arterial and venous thrombosis) and hypofibrinogenemia (which is associated with a higher hemorrhagic risk).

In clinical laboratories, fibrinogen is commonly measured using the Clauss method, a clot-based functional assay. The Clauss assay employs thrombin at concentrations of 35–200 U/mL (typically 100 U/mL), which are applied to pre-diluted plasma samples (typically 1: 10) to minimize interference [7].


https://doi.org/10.3343/alm.2024.0688

The fibrinogen concentration is then determined by interpolating the clotting time with a standard curve prepared with calibrated plasma standards. Commercial Clauss assay reagents typically cover fibrinogen concentrations of 30–1,000 mg/dL, with 5- to 20-fold dilutions if needed.

Clauss assay reagents contain heparin inhibitors that are used to minimize interference at heparin concentrations of 1–2 IU/mL; higher concentrations of heparin and DTIs, such as dabigatran, can cause falsely low fibrinogen test results. Notably, false decreases in fibrinogen levels were reported in patients with coronavirus disease-2019 who were treated with DTIs [18, 19]. In this study, the fibrinogen levels measured using Clauss assay reagents decreased with increasing plasma dabigatran concentrations, whereas ANYFIB.C exhibited no significant interference regardless of the dabigatran concentration used (Fig. 5).

The instability of thrombin further complicates the Clauss assay [9]. Thrombin-based reagents are typically lyophilized for stability, requiring reconstitution before use, which might introduce variability into the assay. In contrast, ANYFIB.C is a liquid reagent that does not require reconstitution, which simplifies its use and reduces potential variability. Moreover, thrombin derived from human or animal plasma might carry risks for pathogen contamination [10, 11], whereas rBat used in ANYFIB.C is pathogen-free and produced under good manufacturing practice-compliant conditions. These features suggest that ANYFIB.C can overcome the limitations associated with Clauss fibrinogen assay reagents.

rBat-induced fibrin clots were not optically detectable at low fibrinogen concentrations (<50 mg/dL) when rBat concentrations of ≥5.00 BU/mL were used (Table 1), although including CMCS enabled optical detection. The inability of rBat alone to form optically detectable clots at low fibrinogen levels might stem from the physical properties of the fibrin clots generated by this enzyme. Thrombin cleaves both the Aα and Bβ chains of fibrinogen, releasing fibrinopeptides A (corresponding to Ala20–Arg35) and B (Gln31–Arg44), respectively, and exposing knobs A and B. Knobs A (Gly–Pro–Arg motif) and B (Gly–His–Arg–Pro motif) can interact with hole a and hole b, which are present in the D-region of other fibrinogen molecules, thereby facilitating protofibril formation and lateral aggregation, respectively [2, 20]. The stability of a fibrin clot is attributable to B–b interactions [21, 22] and interchain (γ–γ, γ–α, or α–α) cross-linking by thrombin-activated FXIIIa [23], which is accompanied by thinner fibrin fibers, enhanced lateral aggregation, and increased fiber density [23]. Unlike thrombin, rBat cleaves only the Aα chain. This cleavage results in a fibrin clot lacking B–b interactions and FXIIIa-induced cross-links critical for the mechanical stability and viscosity of a clot. Including CMCS in the ANYFIB.C reagent enhanced the stability and viscosity of rBat-induced fibrin clots, enabling optical detection at low fibrinogen concentrations. Accordingly, CMCS shortened the clot formation time (CFT) and increased the clot viscosity and stability measured by rotational thromboelastometry (ROTEM) and viscometry (Supplemental Data Table S1 and Supplemental Data Fig. S1). CMCS also enhanced hydrolysis of the thrombin substrate, BIOPHEN CS–01(38), by rBat and facilitated the release of fibrinopeptide A from fibrinogen, thereby accelerating Des-A-fibrin formation (Fig. 3). CMCS is a partially deacetylated derivative of chitin, a natural polysaccharide found in the exoskeleton of crustaceans [24, 25]. Being biocompatible, biodegradable, and nontoxic, chitin and chitosan are widely used for various biomedical applications, especially for polymer scaffold fabrication in tissue engineering [26, 27]. Unlike chitin and chitosan, which are water-insoluble due to their crystalline hydrogen-bonded structures, CMCS is water-soluble, which makes it an ideal functional nontoxic biomaterial [28]. Further studies are needed to elucidate the precise mechanisms whereby CMCS enhances the enzymatic activity of rBat.

In conclusion, our results show that CMCS enhanced the enzyme activity of rBat and stabilized rBat-induced fibrin clots. The ANYFIB.C reagent, comprising rBat and CMCS, helped efficiently measure fibrinogen levels in clinical blood samples. In addition, the characteristic of CMCS may be applied to development of hemostatic pads or fibrin sealants in the future.

## SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/10.3343/alm.2024.0688.

## ACKNOWLEDGEMENTS

None.

## AUTHOR CONTRIBUTIONS

Kim Ju and Park R conceived the study and reviewed and edited the manuscript; Ko E, Song J, and Woo Y performed the experiments, interpreted the results, and drafted the manuscript; Kim Jo and Sohn Y provided technical advice for the experiments and interpreted the results. All authors contributed to writing the manuscript.

## CONFLICTS OF INTEREST

None declared.

## RESEARCH FUNDING

This work was supported by a grant from the Korean Society on Thrombosis and Hemostasis (grant number KSTH 2021-01) and research funding from Soonchunhyang University.

## REFERENCES

1. Tennent GA, Brennan SO, Stangou AJ, O'Grady J, Hawkins PN, Pepys MB. Human plasma fibrinogen is synthesized in the liver. Blood 2007;109:1971–4.
2. Weisel JW. Fibrinogen and fibrin. Adv Protein Chem 2005;70:247–99.
3. Davalos D and Akassoglou K. Fibrinogen as a key regulator of inflammation in disease. Semin Immunopathol 2012;34:43–62.
4. Besser MW and MacDonald SG. Acquired hypofibrinogenemia: current perspectives. J Blood Med 2016;7:217–25.
5. Mackie IJ, Kitchen S, Machin SJ, Lowe GDO, Haemostasis and Thrombosis Task Force of the British Committee for Standards in Haematology. Guidelines on fibrinogen assays. Br J Haematol 2003;121:396–404.
6. Casini A, Blondon M, Lebreton A, Koegel J, Tintillier V, de Maistre E, et al. Natural history of patients with congenital dysfibrinogenemia. Blood 2015;125:553–61.
7. Clauss A. Rapid physiological coagulation method in determination of fibrinogen. Acta Haematol 1957;17:237–46.
8. Castellone DD and Van Cott EM. Laboratory monitoring of new anticoagulants. Am J Hematol 2010;85:185–7.
9. Le Borgne S and Graber M. Amidase activity and thermal stability of human thrombin. Appl Biochem Biotechnol 1994;48:125–35.
10. Burnouf T. Modern plasma fractionation. Transfus Med Rev 2007;21:101–17.
11. Weaver FA, Lew W, Granke K, Yonehiro L, Delange B, Alexander WA, et al. A comparison of recombinant thrombin to bovine thrombin as a hemostatic ancillary in patients undergoing peripheral arterial bypass and arteriovenous graft procedures. J Vasc Surg 2008;47:1266–73.
12. Undas A. Laboratory testing for fibrinogen disorders: from routine investigations to research studies. Semin Thromb Hemost 2024; doi: 10.1055/s-0044-1787725.
13. Mackie I, Casini A, Pieters M, Pruthi R, Reilly-Stitt C, Suzuki A. International council for standardisation in haematology recommendations on fibrinogen assays, thrombin clotting time and related tests in the investigation of bleeding disorders. Int J Lab Hematol 2024;46:20–32.
14. Hantgan R, Fowler W, Erickson H, Hermans J. Fibrin assembly: a comparison of electron microscopic and light scattering results. Thromb Haemost 1980;44:119–24.
15. Braud S, Bon C, Wisner A. Snake venom proteins acting on hemostasis. Biochimie 2000;82:851–9.
16. You WK, Choi WS, Koh YS, Shin HC, Jang Y, Chung KH. Functional characterization of recombinant batroxobin, a snake venom thrombin-like enzyme, expressed from *Pichia pastoris*. FEBS Lett 2004;571:67–73.
17. CLSI. Protocol for the evaluation, validation, and implementation of coagulometers; approved guideline. Volume 28. CLSI H57-A. Wayne, PA: Clinical and Laboratory Standards Institute, 2008.
18. Suzuki S, Tamura T, Hasegawa K, Maeda S, Mori R, Kainuma M, et al. Fibrinogen levels measured by the dry hematology method are lower than those measured by the Clauss method under a high concentration of heparin. Nagoya J Med Sci 2019;81:259–67.
19. Maier CL, Barker NA, Sniecinski RM. Falsely low fibrinogen levels in COVID-19 patients on direct thrombin inhibitors. Anesth Analg 2020;131:e117–9.
20. Geer CB, Tripathy A, Schoenfisch MH, Lord ST, Gorkun OV. Role of 'B-b' knob-hole interactions in fibrin binding to adsorbed fibrinogen. J Thromb Haemost 2007;5:2344–51.
21. Bale MD, Müller MF, Ferry JD. Effects of fibrinogen-binding tetrapeptides on mechanical properties of fine fibrin clots. Proc Natl Acad Sci U S A 1985;82:1410–3.
22. Shimizu A, Schindlauer G, Ferry JD. Interaction of the fibrinogen-binding tetrapeptide Gly-Pro-Arg-Pro with fine clots and oligomers of alpha-fibrin; comparisons with alpha beta-fibrin. Biopolymers 1988;27:775–88.
23. Hethershaw EL, Cilia La Corte AL, Duval C, Ali M, Grant PJ, Ariëns RAS, et al. The effect of blood coagulation factor XIII on fibrin clot structure and fibrinolysis. J Thromb Haemost 2014;12:197–205.
24. Singla AK and Chawla M. Chitosan: some pharmaceutical and biological aspects – an update. J Pharm Pharmacol 2001;53:1047–67.
25. Kast CE, Frick W, Losert U, Bernkop-Schnürch A. Chitosan-thioglycolic acid conjugate: a new scaffold material for tissue engineering? Int J Pharm 2003;256:183–9.
26. Elieh-Ali-Komi D, Hamblin MR. Chitin and chitosan: production and application of versatile biomedical nanomaterials. Int J Adv Res (Indore) 2016;4:411–27.
27. Park BK and Kim MM. Applications of chitin and its derivatives in biological medicine. Int J Mol Sci 2010;11:5152–64.
28. Chen XG, Wang Z, Liu WS, Park HJ. The effect of carboxymethyl-chitosan on proliferation and collagen secretion of normal and keloid skin fibroblasts. Biomaterials 2002;23:4609–14.