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Effect of NDSB on the Fibrinogen Gelation by Thrombin

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Fibrinogen is converted to fibrin gel by the action of thrombin. In our previous study, it has been found that the fibrin gelation was inhibited by the addition of β-cyclodextrin to the mixture of fibrinogen and thrombin. In the present work, the additive effect of non-detergent sulphobetaine (NDSB) was examined. NDSB is known to have an inhibiting effect on the aggregation of proteins. NDSB-195 was used in this work. The gel formation of fibrinogen initiated by the addition of thrombin was greatly affected by the presence of NDSB-195: in the presence of NDSB-195, transparent soft gel was formed different from the usual cloudy stiff gel. This phenomenon was characterized by dynamic light scattering (DLS), enzymatic activity, and HPLC measurements. In the light scattering measurement, scattered light intensity increased rapidly with gelation in a stepwise manner without NDSB. However, when NDSB-195 was added, no remarkable change in the scattered intensity was observed. From the enzymatic activity measurement, it was found that NDSB-195 inhibited about half of the thrombin activity. In the HPLC measurement, the release of fibrinopeptides was inhibited by the addition NDSB-195. These facts suggest that NDSB-195 weakens the thrombin activity, and, in addition, interacts with the fibrinopeptide A and B of fibrinogen, too. As a result, transparent soft gel is formed, and the gelation is delayed.
Key words: Fibrinogen, Thrombin, NDSB, Gelation, DLS, HPLC

1. INTRODUCTION

Fibrinogen is a rod shaped protein with the molecular weight of 3.4×10^5, length of 45 nm and diameter of 9 nm. It is well known that fibrin clots play a fundamental role in a blood coagulation process. Fibrin clot is a gel network composed of fibrin fibers induced by thrombin. Thrombin cleaves two pairs of peptides (fibrinopeptide A and B: FPA and FPB) from α and β chains of the fibrinogen. Removal of these peptides converts fibrinogen into fibrin monomer. Resultantly, an active fibrin monomer having two unmasked binding sites in the central domain that fit to the complementary sites in the domains at the both ends of each molecule appears. Then, two-stranded protofibrils are formed that aggregate laterally to yield fibrin fibers. The protofibrils associate to form fibrin fibers, and fibers join into bundles with larger diameter. Thus, it forms the gel finally [1,2]. In the fibrin gel formation induced by thrombin, it has been reported that appreciable effects appear of the pH change [3,4] or addition of divalent cations [5,6], sphingosin [7] and dextran [8,9]. Dextran interacts with fibrinogen both by reducing the solubility [10,11] and accelerating its polymerization [12]. The growth kinetics of fibrin gel has been studied by the light scattering [13,14,15], turbidity methods [2,8,9] and electron microscopy [1].

In our previous study, it was found that the gelation of fibrinogen was almost inhibited when β-cyclodextrin (β-CD) was added [16]. In addition, the fibrin gelation was strongly inhibited by the addition of β-CD-sulfate. Although β-CD interacts a little also with thrombin (competitive inhibition type interaction), CDs and β-CD-sulfate interact with fibrinogen mainly [17].

Non-detergent sulphobetaine is one of the zwitterionic compounds. Like zwittergents, NDSBs carry the sulphobetaine hydrophilic head group. However, in contrast to usual zwittergents, the hydrophobic group of NDSBs is too short (or small) to form micellar aggregation even at the concentrations as high as 1 M. Hence, they do not behave like detergents. As well as being effective in the extraction [18], solubilization [19] and renaturation [20] of proteins, NDSBs have also been ascertained to enhance both the size and rate of growth [21] of protein crystals.

In the present work, the additive effect of NDSB on the fibrinogen gelation was examined, and it was ascertained that the enzyme reaction and the aggregation (gelation) of the protein were synchronal. NDSB-195 (Fig. 1) was used in this research. The gelation process in the presence of NDSB-195 was analyzed using light scattering. On the other hand, the enzymatic activity of the thrombin was examined using synthetic substrate in order to know how NDSB-195 affects thrombin [22]. Furthermore, the release of FPA and FPB in the gelation process with the addition of NDSB was also examined by using HPLC [23].

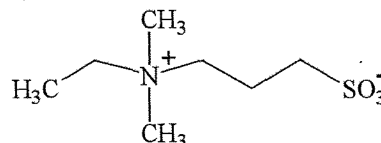

Fig. 1. Molecular structure of NDSB-195

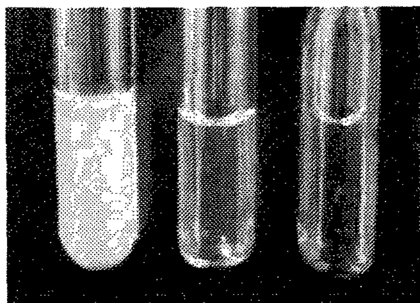

Fig. 2. Turbidity of gel when NDSB-195 was added to fibrinogen-thrombin system (40 hours of elapsed time at 37 °C). The NDSB-195 concentration are 0, 0.25, and 0.5 M from the left to right, respectively

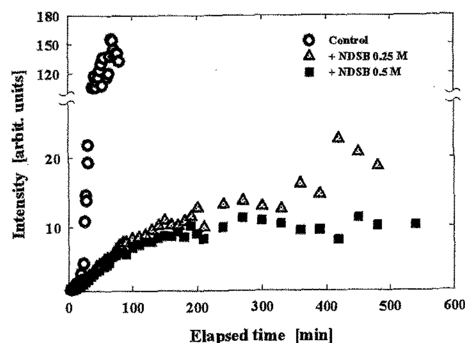

Fig. 3. Time evolution of the scattered light intensity at $\theta = 30^{\circ}$ for the fibrinogen-thrombin system (control) and for the systems in the presence of 0.25 and 0.5 M NDSB-195.

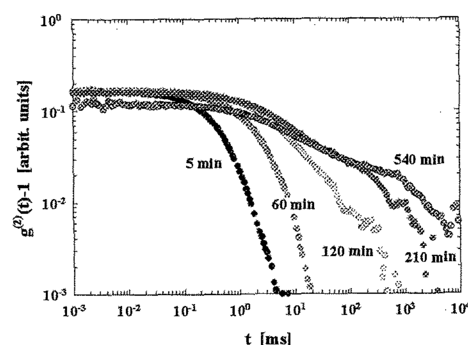

Fig. 4. Double-logarithmic plots of correlation function $g^{(2)}(t)$ - 1 as a function of delay time t in the presence of 0.5 M NDSB-195. Numbers in the figure mean elapsed time.

2. EXPERIMENTAL

Bovine fibrinogen (Sigma-Aldrich Co.) was dissolved in a phosphate buffer saline (PBS: pH 7.4; 136.9 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.10 mM Na_2HPO_4) and this solution was dialyzed against the PBS overnight. Concentration of fibrinogen solution was determined from the absorbance at 280 nm, using an extinction coefficient of 1.51 ml/mg·cm. Bovine thrombin (Wako Pure Chemical) solution was prepared in the PBS at the concentration of 1 NIH units/ml. NDSB-195 (MERCK Co.) solution was prepared by dissolving NDSB-195 in the PBS.

For the light scattering measurements, before mixing the fibrinogen and thrombin solution to start the reaction, fibrinogen solution with NDSB-195 was prepared to make a final concentration of 2.2 mg/ml. Just before the measurements, the both solutions of fibrinogen and thrombin was mixed at the volume ratio of 100:1, and this time of mixing was denoted as the elapsed time t = 0 in the time course of gelation process.

The mixed solution was immediately put into a cylindrical cell having the optical path length of 6 mm by passing it through a membrane filter of 0.2 μm pore size. The preparation was carried out in a clean dry box in order to prevent contamination of impurities. Dynamic light scattering measurements were carried out using a homemade spectrometer and an ALV-5000 multiple-tau digital correlator to obtain the correlation function of scattered light $g^{(2)}(t)$ and the averaged scattered light intensity simultaneously. The decay time distribution function $\tau G(\tau)$ was obtained from $g^{(2)}(t)$ by using CONTIN program. Light source was Ar ion laser with the wavelength λ = 488 nm and the details of the apparatus were described elsewhere [24]. The measurement of $g^{(2)}(t)$ in the time course of gelation were carried out at the scattering angle $\theta = 30^{\circ}$ and were obtained by the homodyne mode.

The thrombin activity measurement was carried out by using synthetic substrate Boc-Val-Pro-Arg-MCA (PEPTIDE INSTITUTE, INC., Japan), and the change of fluorescence intensity was monitored by HITACHI F-4010 fluorophotometer. The thrombin concentration was adjusted so that the final one should become 0.01unit/ml.

High performance liquid chromatography (HPLC) analysis was carried out on a Shimadzu HPLC system (Japan), which included a pump (LC-10Ai), a communication bus module (CBM-10A), and an online degasser (DGU-4A). Analysis was done by a UV-VIS detector (SPD-10Ai). CAPCELL PAK C1 SG300 (4.6 mm i.d. x 150 mm, Shiseido, Japan) was used as the analytical column. Fibrinopeptides were separated with a linear acetonitrile gradient solution according to Kehl et al. [25] with the following modifications: instead of ammonium acetate buffer, 0.1 % TFA or 0.1 % TFA in acetonitrile were used for solvents A or B, respectively. The gradient was run from 5 to 17.5 % solvent B in 25 min with a flow rate of 1.0 ml/min. Detection was carried out at 215 nm.

All the measurements were performed at the temperature of 37 °C.

3. RESULTS AND DISCUSSION

Generally, the gel (control gel) formed in the fibrinogen-thrombin system is very cloudy. But, when NDSB-195 was added, a transparent gel was formed (Fig. 2). And, it was much softer than the control gel. There is a report that a fibrin gel becomes transparent due to the increase in pH and ionic strength [4]. However, since NDSB is zwitterionic substance, it does not the case for the present one.

Figure 3 shows the scattered light intensity as a function of the elapsed time with the addition of

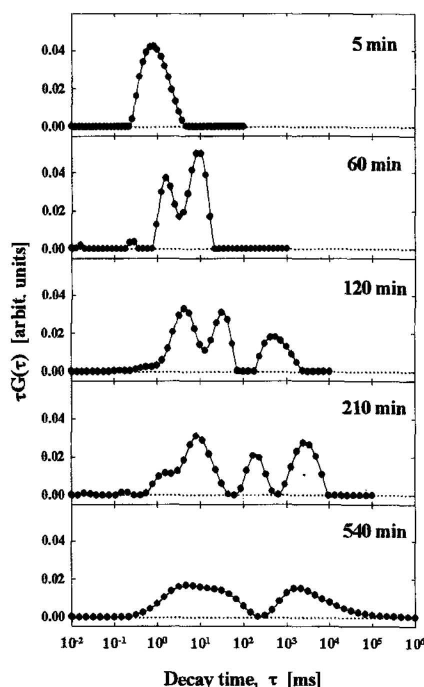

Fig. 5. Typical result of time evolution of decay time distribution function $\tau G(\tau)$ in the presence of 0.5 M NDSB-195 to the fibrinogen-thrombin solution.

NDSB-195. In the fibrinogen-thrombin system, the scattered light intensity exhibited rapid increase with the progress of fibrinogen gelation [26]. However, in the present case with NDSB-195, such a rapid increase of the scattered intensity was not observed at all. The increase of scattered light intensity with 0.5M NDSB-195 was only about 1/15 of the fibrinogen-thrombin system at the elapsed time of 10 hours. Such a very slow and tiny increase of the scattered light intensity in the system with NDSB-195 is due to that the density fluctuation became very small, and the system was more uniform than the fibrinogen-thrombin system. This result was very similar to that when β-CD was added [16].

Figure 4 shows the double-logarithmic plots of the correlation function $g^{(2)}(\tau)$ - 1 as a function of the decay time τ in the solution where NDSB-195 was added by 0.5 M. The $g^{(2)}(\tau)$ - 1 shows a characteristic decay behavior similar to the usual polymer solution with very broad decay time distribution for the early period of the gelation. And, with the increase of elapsed time, $g^{(2)}(\tau)$-1 became to show a slowing down of the decay. The initial value corresponding to the extent of coherence of detection was decreased slightly. It is suggested that a decrease in the initial value is small because the structure of the gel is uniform, although this result corresponds to the occurrence of gelation.

Figure 5 shows the time evolution of the decay time distribution function $\tau G(\tau)$ with the addition of NDSB-195. At the initial state, $\tau G(\tau)$ showed a unimodal distribution in the short decay time region. Profile of $\tau G(\tau)$ of fibrinogen solution without thrombin is almost the same as that at the initial state. The peak of $\tau G(\tau)$ at the initial state shifted to a longer decay time

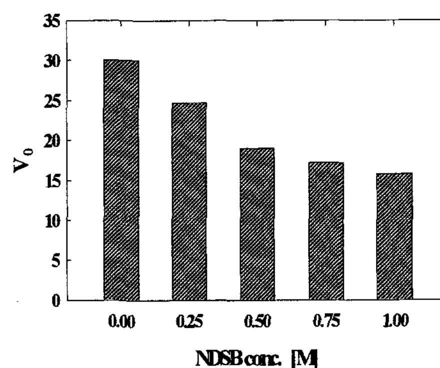

Fig. 6. Effect of NDSB-195 on the cleavage of synthetic substrate by thrombin.

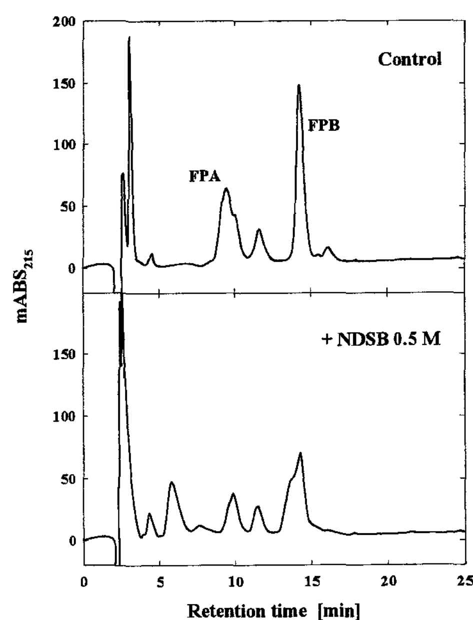

Fig. 7. HPLC chromatography (absorbance at 215 nm vs. retention time).

region with increasing elapsed time. This behavior is parallel to a typical gelation process. Then, it was examined whether NDSB-195 interacts with fibrinogen and thrombin.

Thrombin activity using the synthetic substrate was examined by monitoring the fluorescence intensity in the time course after the addition of thrombin to the substrate solution. The relationships of the initial slope of the fluorescence intensity against time V_0 was obtained. Figure 6 shows the influence of NDSB-195 on the enzymatic activity of thrombin. When NDSB-195 was added by 0.5 M, V_0 became smaller by about 2/3. In our previous studies, it has been found that the enzymatic activity of several enzymes was not inhibited even if NDSB was added (unpublished results).

On the other hand, the release of FPA and FPB in the fibrinogen-thrombin system with or without NDSB-195 was investigated using the HPLC measurement (Fig. 7). In the fibrinogen-thrombin system, three peaks appeared

in the chart, and the distinct sequences were determinable for the first and third peaks. The first and third peaks are the peaks of the FPA and FPB, respectively [27]. However, the release of FPA and FPB fairly decreased for the sample with NDSB-195.

From the result of HPLC and thrombin activity measurements, NDSB-195 is likely to interact with thrombin. Decrease of V_0 with the addition of NDSB should be due to the decrease of V_{max} or K_m of enzyme reaction. If the decrease of V_{max} is the case, the amount of FPA and FPB release might be unchanged at sufficiently long elapsed time. Another possibility is the difficulty in the sample preparation. The samples for the HPLC measurement were generally prepared from the supernatant solution by boiling the gelled samples (for degeneration or breaking of the aggregation) and by ultracentrifugation of them. But, such a procedure does not give clear precipitation and supernatant for the sample with NDSB, because NDSB-195 works to inhibit the aggregation of fibrinogen. Therefore, it was probable that the decrease of FPA and FPB release might be due to incomplete separation of FPA and FPB into the supernatant. Therefore, we applied the precipitation method by the addition of acetone instead of boiling, and the separation was achieved sufficiently. Then this possibility could be ruled out. Then, the result of Fig. 7 suggests that the decrease of K_m might occur in the present case. However, it is difficult to conclude which is valid.

4. CONCLUSION

According to the present work, it is suggested that NDSB-195 delays the gelation by acting on the thrombin and forms a uniform gel (transparent and soft gel) with weakening the aggregation of fibrin(ogen). The effects and functions of NDSBs differ for various proteins [28]. Only one type of NDSBs (NDSB-195) was investigated in the present work and remarkable effects were observed, and there are different possibilities of effects and functions for other NDSBs.

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