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Effect of Saccharides on the Fibrinogen-Fibrin Conversion by Thrombin

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Fibrinogen is converted to fibrin gel by the action of thrombin. This phenomenon is the last stage in the cascade of blood coagulation. In our previous study, it was found that the gelation was inhibited by adding 10 mg/ml β-Cyclodextrin (β-CD) to the mixture of fibrinogen and thrombin. It was ascertained that the addition of heparin (0.1 mg/ml) inhibits the blood coagulation, the concentration of heparin was that used in the clinical field. The effect of β-Cyclodextrin-sulfate (β-CD-S), which mimics heparin, was also investigated. β-CD-S was added (18.2 mg/ml) so that it becomes the same content in the glucopyranose unit as β-CD. Scattered light intensity and the decay time distribution function were obtained by the dynamic light scattering as a function of the elapsed time after the addition of saccharides. In all cases, gelation did not occur. From the Lineweaver-Burk plot for the enzymatic activity measurements, it was found that β-CD strongly inhibited the thrombin activity as a noncompetitive inhibition. For the samples with β-CD-S and heparin, the almost no inhibition was observed. In the HPLC measurements, it was found that the release of fibrinopeptide was not affected by the addition of β-CD so much, but it was almost inhibited by the addition of β-CD-S. These facts suggest that β-CD interact with thrombin and β-CD-S with fibrinogen mainly.
Key words: Cyclodextrin, Fibrinogen, Thrombin, Gelation, Dynamic Light Scattering, HPLC, Synthetic Substrate

1. INTRODUCTION

Fibrinogen is a rod shaped protein with the molecular weight of 3.4×10^5. The final stage of blood coagulation cascade is the fibrinogen-fibrin conversion induced by thrombin. This reaction is initiated by the removal of fibrinopeptide A and B (FPA and FPB) by thrombin and is terminated with the formation of a three-dimensional gel network (fibrin gel). Formation of the fibrin gel proceeds in a stepwise manner. On the activation, fibrinogen molecules polymerize in end-to-end manner forming protofibrils and by side-by-side association forming gel network. The protofibrils associate to form fibrin fibers and fibers join into bundles with larger diameter. Then, it finally forms the gel [1,2].

The growth kinetics of fibrin gel has been studied by the light scattering [3,4,5], turbidity methods [2,6,7] and electron microscopy [1]. On the other hand, in fibrinogen to fibrin gel conversion by thrombin, it was reported that appreciable effects appear of the pH change [8] or addition of divalent cations [9,10], sphingosin [11] and dextran [6,7]. Dextran interacts both with fibrinogen reducing solubility [12,13] and with fibrin accelerating its polymerization [14]. Moreover, it was recently found that the gelation is enhanced with the increase of the molecular weight of added dextran.

In our previous study, it was found that the gelation of fibrinogen was almost inhibited when β-CD was added [15]. Cyclodextrin has a doughnut-like structure and the central hole provides a hydrophobic environment. Therefore, various residues and molecules can be taken into this hole. Because of these novel characteristics, interesting inclusion phenomena have been observed

In recent years, the dextran sulfate salt has become to be used clinically, because it shows blood anticoagulant action. In the present work, the effect of addition of β-CD-S to the fibrinogen gelation was examined, because β-CD-S was a candidate of heparin mimics [16]. The gelation process with adding these saccharides was analyzed using light scattering. On the other hand, the enzymatic activity of the thrombin was examined in order to know how these saccharides affect to fibrinogen and/or thrombin using synthetic substrate [17]. Furthermore, the release of FPA and FPB in the gelation process with the addition of saccharides was also examined using HPLC [18].

2. EXPERIMENTAL

Bovine fibrinogen (Sigma-Aldrich Co.) was dissolved in a physiological saline solution (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 physiological saline solution 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 (Mochida Co., Japan) solution was prepared in the physiological saline solution at the concentration of 1 NIH units/ml. The β-CD, β-CD-S, and heparin (Sigma-Aldrich Co.) solution was prepared by dissolving them in the similar physiological saline solution. 1.82 times of β-CD-S was added in order to keep the same content in the glucopyranose unit as β-CD. Heparin was prepared at the concentration used in clinical field (0.1 mg/ml).

For the light scattering measurements, before mixing the fibrinogen and thrombin solutions to start the reaction, fibrinogen solution with saccharides 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

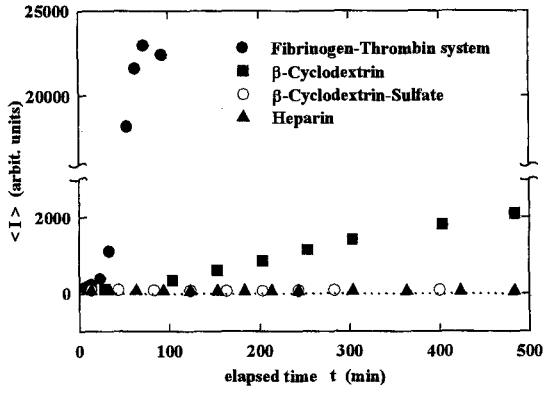

Fig. 1. Time evolution of the scattered light intensity at θ =30° for the fibrinogen-thrombin system and for the system in the presence of 10 mg/ml β-CD, 18.2 mg/ml β-CD-S and 0.1 mg/ml heparin.

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 < I > simultaneously. The decay time distribution function G(τ) was obtained from $g^{(2)}(\tau)$ by using CONTIN program. Light source was Ar ion laser with the wavelength λ = 488 nm and the details of the apparatus was described elsewhere [19]. The measurement of $g^{(2)}(\tau)$ in the time course of gelation were carried out at the scattering angle θ = 30 ° 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. [20] 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.

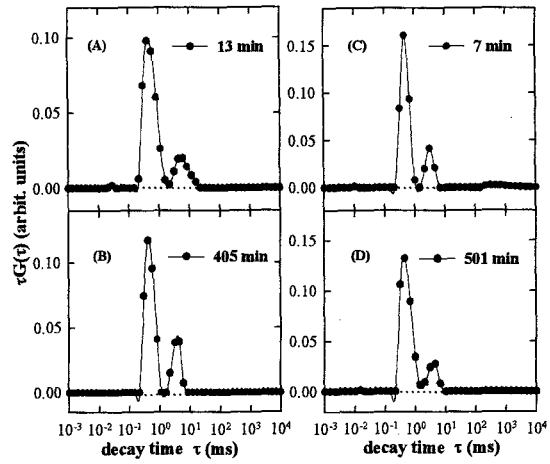

Fig. 2. Time evolution of the decay time distribution function τG(τ) in the presence of 18.2 mg/ml β-CD-S (A),(B) and 0.1 mg/ml heparin (C),(D). (A) and (C) are at the initial stage of the reaction, and (B) and (D) are at several hours after the reaction start.

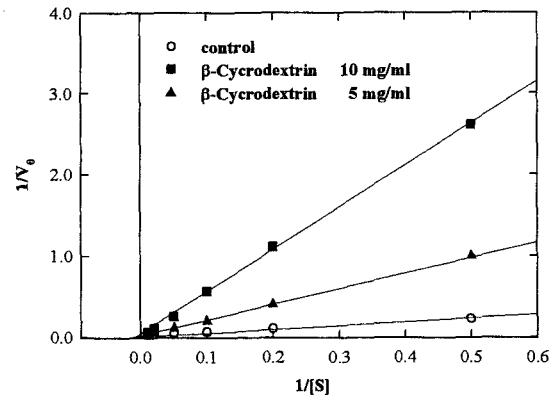

Fig. 3. The Lineweaver-Burk plot with the addition of β-CD (10 or 5 mg/ml) to the synthetic substrate-thrombin system.

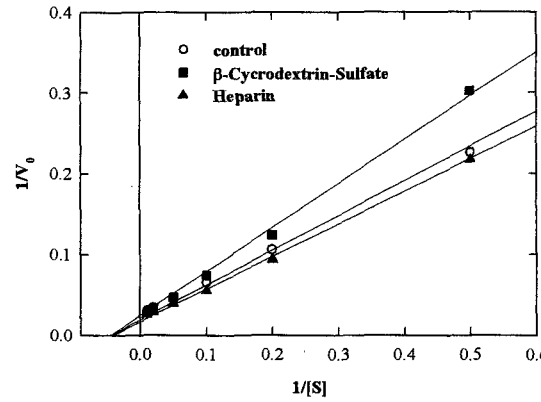

Fig. 4. The Lineweaver-Burk plot with the addition of β-CD-S (18.2 mg/ml) and heparin (0.1 mg/ml) to the synthetic substrate-thrombin system.

3. RESULTS AND DISCUSSION

Figure 1 shows the scattered light intensity < I > as a function of the elapsed time t with the addition of various saccharides. In the fibrinogen-thrombin system, the scattered light intensity exhibited a low value for t < 40 min, and rapidly increased at t > 40 [21]. However, in case of the sample with 10 mg/ml β-CD, such a rapid increase of the scattered intensity was not observed [15]. Furthermore, in the samples of β-CD-S (18.2 mg/ml) or heparin (0.1 mg/ml), none of the increase in the scattered light intensity was observed. In the samples with β-CD, β-CD-S and heparin, the gelation did not occurred. It is known that heparin shows the anticoagulant action with promoting the activation of the antithrombin III (AT III) in the blood coagulation process. It was ascertained that heparin inhibits the gelation, even though AT III does not exist in present experimental condition. Sulfate group seems to affect the fibrinogen-fibrin conversion effectively, because the β-CD-S also suppresses the gelation.

Figure 2 shows the time evolution of the decay time distribution function $\tau G(\tau)$ with the addition of β-CD-S and heparin. For those solutions of β-CD-S or heparin, the change as observed in β-CD could not be observed. Any noticeable change was detected on both decay time distribution at the initial stage of reaction and at several hours after the start of reaction. Only a small peak has appeared in the longer time side of the decay time other than the peak, which is equivalent to a fibrinogen molecule. This small peak seems to correspond to the protofibril, and its peak does not grow up. Gelation does not occur there, because the activity of the thrombin is inhibited: that is, the release of FPA and FPB is inhibited as mentioned below.

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 and the substrate concentration [S] were obtained. Then, a double-reciprocal plot (Lineweaver-Burk plot) was done in V_0 and [S]. Figure 3 shows the Lineweaver-Burk plot for the sample with the addition of β-CD (10 or 5 mg/ml). From this figure, it was found that β-CD strongly inhibits the thrombin activity. The type of inhibition was not uncompetitive inhibition, because the $1/V_0$ value increase with the increase in the β-CD concentration and the linear regression line intersects almost on the ordinate. However, the differentiation between the competitive and noncompetitive inhibition is not definite. Figure 4 shows the Lineweaver-Burk plot for the solutions with β-CD-S and heparin. Slight inhibiting effect was observed, when β-CD-S was added, but in case of heparin, there was hardly any change, although these effects are only a little.

The release of FPA and FPB at the Fibrinogen-thrombin system with or without saccharides was investigated using the HPLC (Fig. 5). In the fibrinogen-thrombin system, there appeared 4 peaks as observed in the chart, and distinct sequences were determinable for the first and third peaks. No definite sequence could be detected from other 2 peaks. The first and third peaks are the result of the amino acid sequence of the EDGSDPPSGDFLTEGGGVR and

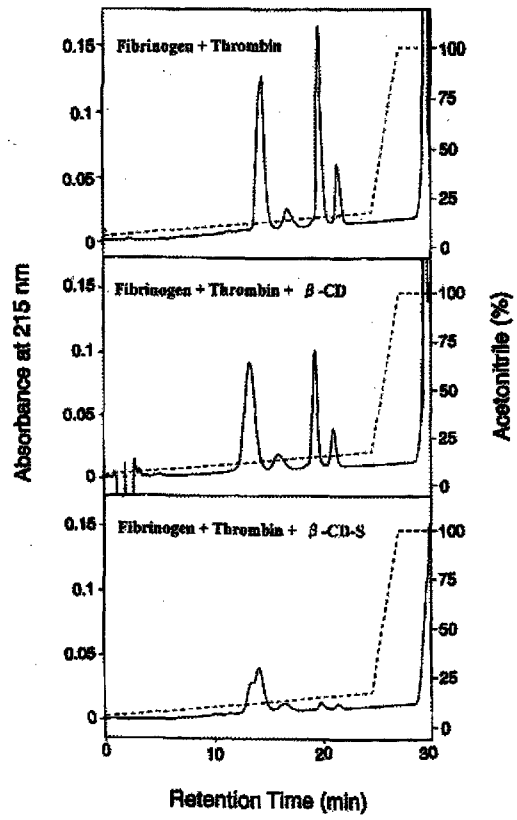

Fig. 5. HPLC chromatography (absorbance at 215 nm vs. retention time); solid line. The dotted line is shown the proportion of flow acetonitrile.

TDYDEGQDDRPKVGLXA, respectively. The first peak agreed with FPA perfectly. However, the third peak has a sequence where two amino acid residues of N-terminal lacked from the reported sequence of FPB [22]. The reason of this is unknown at present.

The results for the sample with the addition of β-CD to the fibrinogen-thrombin system confirmed the release of FPA and FPB. It was considered that β-CD couldn't suppress the thrombin activity sufficiently. This might be related to the fact that the concentration of thrombin is ten times larger than that in the light scattering experiments in the HPLC measurements.

However, the release of FPA and FPB could not be detected for the sample with β-CD-S. From the result of HPLC and thrombin activity measurements, β-CD-S seems to interact with fibrinogen and prevent the gelation by protecting the FPA and FPB release.

The investigations for α- and γ-CD are not reported here. It should be mentioned that the gelling behavior is dependent upon the suppliers of the reagent.

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