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Effects of Mono-, Oligo- and Polysaccharides on Fibrin Gelation

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The effects of addition of mono-, oligo- and polysaccharides to the fibrin gel formation process were systematically investigated by using static and dynamic light scattering combined with turbidity measurements. Glucose and oligosaccharides, such as maltose, maltotetraose and maltoheptaose, retarded the fibrin gelation, and the retarding effect becomes significant with the decrease in the number of glucopyranose units composing the saccharides. The fibrin fibers formed in the presence of those saccharides are thin and loose ones. In contrast, dextran which is a polysaccharide accelerates the gelation with the increase in its molecular weight. The fibrin gel network in the presence of dextran was examined by confocal laser scanning microscopy (CLSM). The spatial distribution of dextran molecules coincides well with that of fibrin fibers, indicating that dextran molecules are assembled along the fibrin fibers. In addition, the number density of fibrin fibers in the presence of dextran is fairly less compared to that without dextran. The enzymatic activity of thrombin examined by using a synthetic substrate showed no effect of saccharides, glucose to dextran. That is, these saccharides do not interact with thrombin, but interact with fibrinogen and/or fibrin.
Key words: Fibrinogen, Saccharides, Gelation, Dynamic light scattering, Turbidity

1. INTRODUCTION

The final stage of blood coagulation reaction is the fibrinogen-fibrin conversion induced by thrombin. This reaction is initiated by the release of fibrinopeptide A and B (FPA and FPB) by thrombin and results finally in the formation of a three-dimensional gel network (fibrin gel). The growth kinetics of fibrin gel has been studied by the light scattering [1-4], turbidity [5-7], X-ray crystallography [8] and real space observation by confocal laser scanning microscopy (CLSM) [4,9]. The formation of fibrin gel proceeds in a stepwise manner: activation of fibrinogen to originate the fibrin monomer and half staggered axial polymerization of fibrin monomers (protofibril formation) in the first step, and side-by-side association of protofibrils (fiber growth) resulting the gel formation in the succeeding step. [5,10]

In the fibrinogen-to-fibrin gel conversion by thrombin, appreciable effects have been found in the change of pH [11], ionic strength [12], the addition of divalent cations [13,14], and the addition of saccharides. Dextran, which has been used as a plasma expander for more than 50 years, interacts both with fibrinogen reducing solubility [15,16] and with fibrin accelerating its polymerization [17]. The accelerating effect increases with the increase in the molecular weight of added dextrans. Recently, it has been found that cyclodextrins (particularly β-cyclodextrin) retarded extremely the fibrin gel formation [18,19]. On the other hand, other saccharides, such as glucose and maltose, exist as energy source in vivo and its solutions are used clinically as a transfusion. Especially, glucose is the most prevalent monosaccharide unit in oligo- and polysaccharides, and coexists in blood, too. The concentration level of glucose in blood is *ca.* 1.1 mg/ml in a normal case and rises upto *ca.* 5.0 mg/ml in a diabetic. Thus, it is very worthy to investigate the effect of additions of mono- to polysaccharides on the fibrin gel formation systematically.

In the present study, we examined the additive effects of linear saccharides, glucose to dextran, on the process of fibrin gel formation by using light scattering combined with turbidity measurements. Furthermore, the effect of those saccharides on the enzymatic activity of thrombin was examined by using a synthetic substrate. The fibrin network in the presence of dextran was observed by CLSM in order to reveal the spatial distribution of dextran in the fibrin gel.

2. EXPERIMENTAL

Bovine fibrinogen (clottability 97%, Sigma -Aldrich) was dissolved in a physiological saline solution (pH 7.4 ; 136.9 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4) and this solution was dialyzed thoroughly against the

physiological saline solution overnight. Concentration of fibrinogen was determined from the absorbance at 280 nm using an extinction coefficient of 1.51 ml/mg·cm.

Bovine thrombin (Wako Pure Chemical Industries) solution was also prepared in the physiological saline solution. Special care was paid to avoid unnecessary adsorption of thrombin to the vessels. Fibrinogen solution was prepared to make a final concentration of 2.2 mg/ml, and the final thrombin concentration was 0.01 NIH units/ml in all the measurements.

The saccharides used in this experiment were glucose, maltose, maltotetraose, maltoheptaose (Sigma-Aldrich), dextran (nominal molecular weight = 77000, 9500 [Sigma-Aldrich] and 4300 [Sowa Science Corporation]), and the solutions were prepared by dissolving them in the physiological saline solution. Those samples were all reagent grade, and the final concentration was adjusted to 10 mg/ml.

Dynamic light scattering measurements were carried out using a homemade spectrophotometer and an ALV-5000 multiple-tau digital correlator to obtain the correlation function of scattered light $g^{(2)}(\tau)$, and the averaged scattered light intensity was monitored simultaneously. The decay time distribution function $\tau G(\tau)$ was obtained from $g^{(2)}(\tau)$ by using CONTIN program. Light source was He-Ne laser with the wavelength of 632.8 nm, and the details of the apparatus was described elsewhere [20]. Measurements of $g^{(2)}(\tau)$ in the time course of gelation were carried out at the scattering angle of 30° using the homodyne mode.

Turbidity measurements were performed using U-2000A spectrophotometer (Hitachi). Wave length dependence of turbidity was obtained scanning over 400 to 800 nm at every 5 minutes.

CLSM observations were carried out by using MRC-1024 (BioRad). Texas Red-labeled dextran (Molecular Probes) was incorporated into 0.5 % of total dextran in the fibrinogen-dextran mixed solution as a fluorescent probe. Completely gelled samples were treated by fluorescein isothiocyanate (FITC) in order to stain fibrin fibers, and then washed by the physiological saline solution to remove the free dyes. To obtain the confocal images, laser line were used with the wavelengths of 488 and 543 nm to excite FITC and Texas Red, respectively.

The thrombin activity measurements were carried out by employing the synthetic substrate Boc-Val -Pro-Arg-MCA (Peptide Institute). The change of fluorescence intensity at 460 nm (emission) from MCA (4-methyl-coumaryl-7-amide) excited by 380 nm incident light was monitored. The measurements were done by using F-4010 fluorophotometer (Hitachi).

All the measurements were carried out at 37 °C (±0.05 °C). In the measurements of temporal growth of the fibrin gel, the time of mixing of thrombin with the fibrinogen solution was assigned as the elapsed time 0.

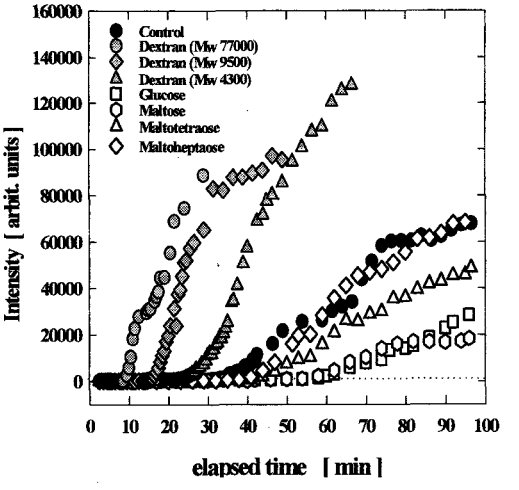

Fig. 1. Temporal evolution of the scattered light intensity at the scattering angle 30° for the fibrinogen-thrombin systems with various saccharides. Control represents the fibrinogen -thrombin system without any saccharides.

3. RESULTS AND DISCUSSION

Figure 1 shows the effect of addition of various saccharides on the temporal evolution of scattered light intensity. The increase of scattered light intensity is due to the aggregation of fibrin, protofibril formation, lateral aggregation of protofibrils and network formation. Especially lateral aggregation and network formation result in a very large increase of intensity. In the presence of dextran, the acceleration effect of the fibrin gelation was confirmed as compared to the control, and the gelation is enhanced with the increase in the molecular weight of added dextran. On the other hand, retardation of gelation occurs with the addition of mono- and oligosaccharides, and the extent of retardation decreases with the increase of the number of glucopyranose units composing the saccharides. These findings suggest that the fibrin gel formation is affected by the interaction of the saccharides with fibrinogen and/or thrombin. Furthermore, the effects strongly depend on the molecular weight of saccharides.

The temporal growth of the decay time distribution function $\tau G(\tau)$ with the addition of glucose is shown in Fig. 2 as a typical result. $\tau G(\tau)$ showed a unimodal distribution in the short decay time region at the elapse time t = 4 min, and its peak coincided with that obtained in the fibrinogen solution without thrombin. At t = 20 min, the second peak appeared in a slower decay time region attributable to the formation of protofibrils. The peak of $\tau G(\tau)$ sifted to a longer decay time region and became to be wider with increasing t. These behaviors of $\tau G(\tau)$ indicate the progress of protofibril formation. Essentially the same results were obtained for the addition of other saccharides, except for the growth rate of

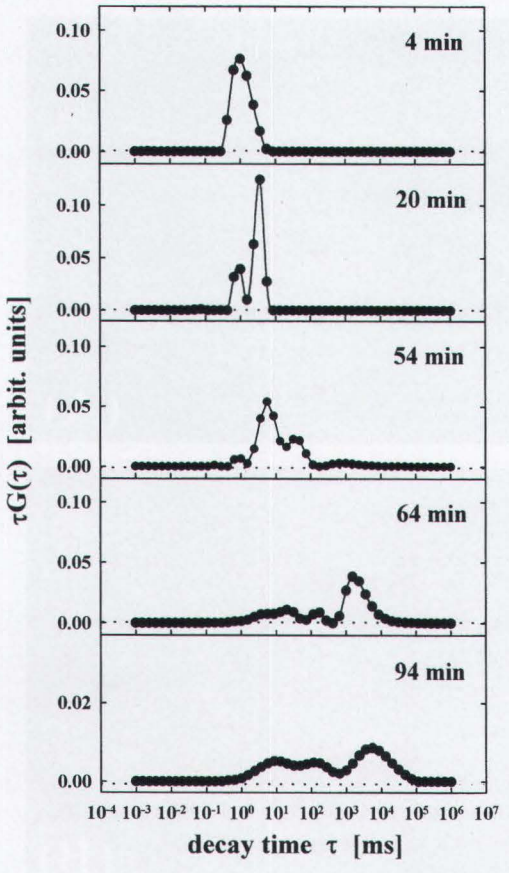

Fig. 2. Temporal growth of the decay time distribution $\tau G(\tau)$ for the fibrinogen-thrombin system with glucose. The elapsed time is indicated at the right margin in each panel.

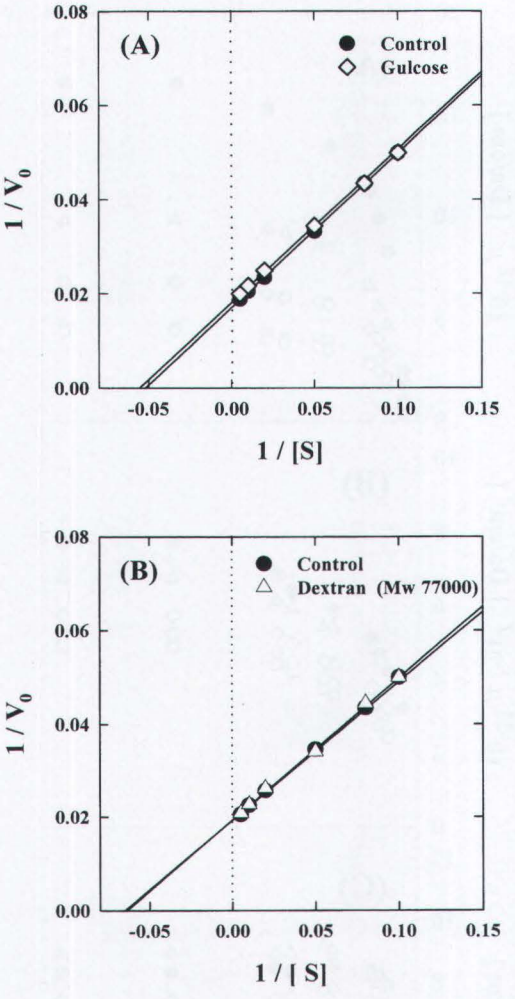

Fig. 3. Lineweaver-Burk plot for the synthetic substrate-thrombin system with glucose (A) and dextran (B). V_0 and [S] represent the initial rate of thrombin-catalyzed reaction and the substrate concentration, respectively.

the decay time distribution.

Figures 1 and 2 suggest the possible interaction of saccharides with fibrinogen and/or thrombin. In order to verify such a possibility in more detail, inhibition effect of thrombin activity by the addition of saccharides was examined. Typical results are shown in Fig. 3 as a Lineweaver-Burk plot for the addition of glucose (A) and dextran with a nominal molecular weight of 77,000 (B). The addition of either glucose or dextran does not vary the relationship between the reciprocal of the initial rate of thrombin-catalyzed reaction V_0 and the substrate concentration [S] at all and the similar results were obtained for all other saccharides. That is, the saccharides (as far as those examined in the present work) do not interact with thrombin, but interact with fibrinogen and/or fibrin.

Time-resolved scanning measurements of turbidity τ were carried out over the wavelength λ of 400 to 800 nm in order to estimate the mass per length ratio μ, the radius r and the density $\mu / \pi r^2$ of fibrin fiber assembly. The analyses were made by using the following equation according to the procedure employed by Carr et al [6,7].

$$(c/\tau\lambda^3) = A/\mu + B(r^2/\mu)\lambda^{-2} \qquad (1)$$

where c is the fibrinogen concentration, and A and B are the constants. Equation (1) implies that a plot of $c/\tau\lambda^3$ vs λ^{-2} should give a straight line, whose intercept and slope are proportional to the reciprocal of μ and $\mu / \pi r^2$, respectively. It should be noteworthy that the μ value corresponds to the extent of lateral aggregation of protofibrils.

Figure 4 shows a growth of μ (A), $\mu / \pi r^2$ (B) and r (C) and with the elapsed time. The amount of the typical experimental error is shown for the case of control. μ value for the control sample increases abruptly around at 35 min, almost the same elapsed time that the scattered light intensity increases abruptly shown in Fig. 1, and reaches the plateau level of *ca.* 1.7×10^{13} Da/cm. For the samples with the addition of mono- and oligosaccharides, μ values reach appreciably less plateau level, and both $\mu / \pi r^2$ and r values are fairly less than those obtained for the control sample. These results indicate clearly that the fibrin fibers composing gel network in the

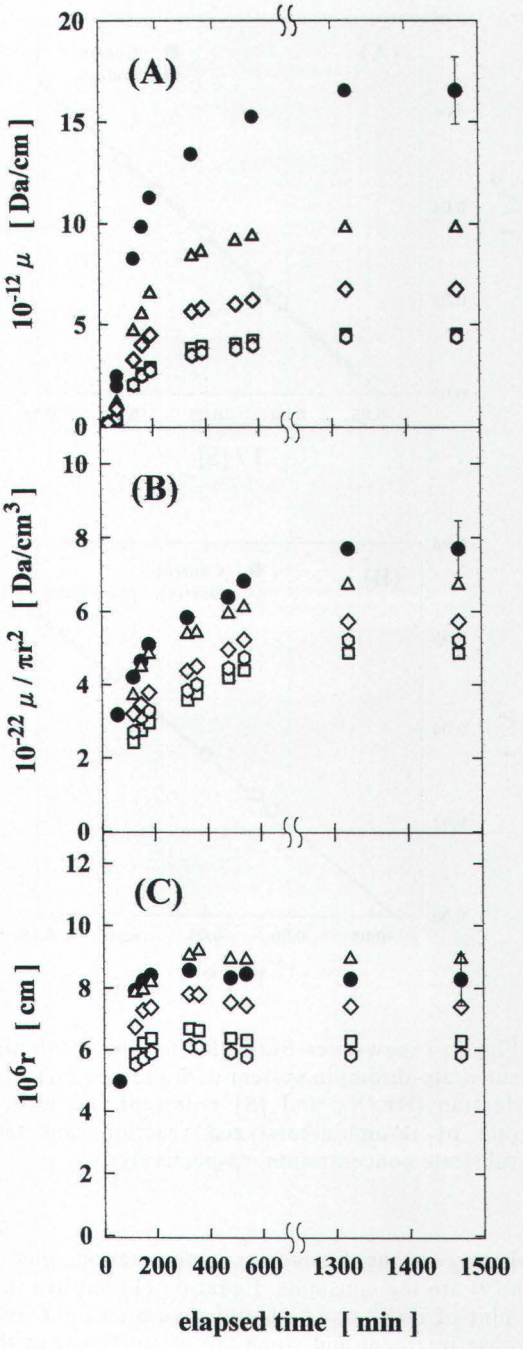

Fig. 4. Temporal variation of mass/length ratio μ (A), density $\mu/\pi r^2$ (B) and radius r (C) of fibrin fiber assembly. Meanings of the symbols are the same as those in Fig.1.

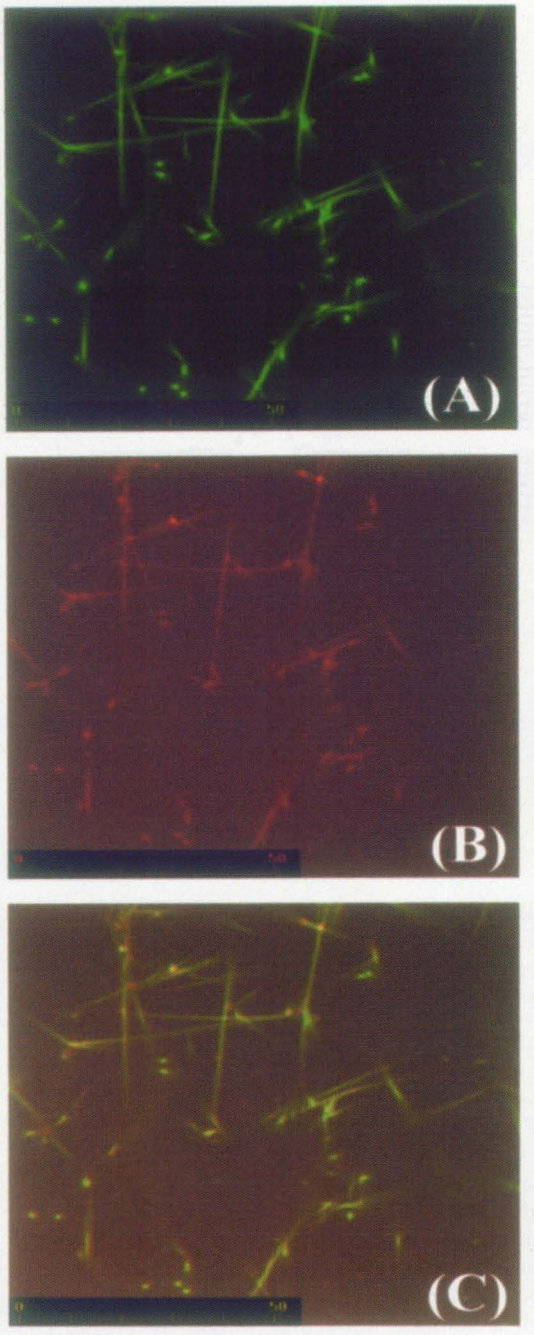

Fig. 5. CLSM images of fibrin assembly. The conditions of observations are (A): emission of FITC-labeled fibrin only, (B): emission of Texas Red-labeled dextran only, (C): emission of both FITC-labeled fibrin and Texas Red-labeled dextran. The scale length in the panel is 50 μm.

presence of mono- and oligosaccharides are thin and loose, since lateral aggregation does not proceed sufficiently. On the other hand, it has been reported by Carr et al. that the presence of dextran resulted in the production of thick (increase in radius) and loose (decrease in density) fibrin fiber assemblies through enhancement of the lateral aggregation. However, it has not been clarified whether dextran is located on the local surface of the fiber or distributed throughout the fiber and what interaction causes such a variation.

The spatial distribution of dextran molecules in the fibrin gel was observed by CLSM as shown in Fig. 5. Fibrin and dextran molecules are

expressed by FITC in green (emission wave length of 520 nm) and Texas Red in red (emission wavelength of 615 nm), respectively. It should be noted that CLSM images are the fluorescence images not the real ones, and the fiber radius is not able to be evaluated by them. The spatial distribution of dextran molecules coincides well with that of fibrin molecules, indicating that dextran molecules locate along the fibrin fibers. Furthermore, the number of fibrin fibers with dextran is fairly less as compared to that without dextran (image is not shown). These facts agrees well with the large mass per length ratio obtained by turbidity measurements. Therefore, it is suggested that saccharides should interact with fibrin fibers but the molecular length of saccharides is important to result in the enhancement of lateral aggregation, and that saccharides which have sufficient molecular length to be able to interact with several fibrin fibers work as a bulky connecting agent. Therefore, mono- and oligosaccharides resulted in thin and loose fibrin fibers in contrast to the case of polysaccharide dextran. The molecular weight dependence of the enhancing effect of dextran to the gelation is thus well elucidated. At the present stage, it can not be distinguished definitely yet which domains of fibrin(ogen) molecule, D domain or E domain, interact with saccharides effectively. Such a study is quite desirable for more detailed study.

4. CONCLUSION

In conclusion, the additive effects of various linear saccharides, glucose to dextran, on fibrin gelation were examined by the dynamic light scattering, turbidity, and CLSM measurements. The fibrin gelation is retarded by the addition of oligosaccharides, and the retardation effect become more significant with the decrease in the number of glucopyranose units composing the saccharides. Furthermore, the fibrin fibers formed in the presence of those saccharides are thin and loose. In contrast, dextran accelerates the fibrin gelation with increase in its molecular weight. The spatial distribution of dextran molecules coincides well with that of fibrin molecules, indicating that dextran molecules locate along the fibrin fibers. There is no effect of the saccharides, glucose to dextran, on thrombin activity. That is, these saccharides do not interact with thrombin, but interact with fibrinogen and/or fibrin. In order to enhance the lateral aggregation of fibrin fibers effectively the chain length of saccharides is important factor. These results will give useful information for the blood coagulation in a diabetic and a dextran transfusion recipient.

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