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Stereoisomer Effect of Disaccharides on the Interaction with Fibrinogen

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Stereoisomer effects of the addition of disaccharides to the fibrin gel formation process induced by serine protease, thrombin, were investigated systematically by using light scattering combined with turbidity measurements. Three disaccharides, maltose, isomaltose, and cellobiose, were of glycoside-glucose type, and the temporal growth of fibrin gel formation in the presence of them were compared with each other. Maltose and cellobiose retarded the fibrin gelation, and the resultant fibrin fibers were thin and loose ones. In contrast, the result in the presence of isomaltose agreed with the control (without disaccharides), and the fibrin gel formation and the characteristics of fibrin fibers were not affected. According to the HPLC measurements, the release of fibrinopeptides A and B were found not to be affected by the addition of those disaccharides so much. Disaccharides do not interact with thrombin, but do with fibrinogen and/or fibrin.
Key words: Fibrinogen, Gelation, Saccharides, Stereoisomer Effect, Light Scattering,

1. INTRODUCTION

Fibrinogen is a major component functioning in the process of blood coagulation, thrombosis, and hemostasis. Fibrinogen molecule is a rod-shaped dimeric glycoprotein, with each monomer composed of three polypeptides: the Aα (610 amino acid residues), the Bβ (461 residues), and the γ (411 residues) chains. 29 disulfide bonds to form stiff molecular assembly interconnect those six chains (the numbers of residues are for human-fibrinogen [1,2]). Although the primary structure of the protein has been well known already, detailed structural analysis has now been achieved well. According to electron micrograph measurements, fibrinogen has an elongated trinodular structure with three globular domains connected by two spacer arms. The central globular E domain contains the dimmer interface along with the amino terminal of all six chains. The D domains at both end regions consist of the carboxy termini of the Bβ and γ chains. The Aα chain carboxy termini extend freely from the D domains and seem to interact with the central E domain at the terminal regions.

The final stage of blood coagulation cascade is the fibrinogen-fibrin conversion induced by serine protease, thrombin. Thrombin cleaves arginine-glycine linkages at the N termini of Aα and Bβ chains, and releases fibrinopeptides FpA and FpB. The exposed sites of Aα and Bβ chains work effectively in the fibrinogen-fibrin conversion and protofibril formation, lateral aggregation, and fibrin gel formation proceeds successively. Growth kinetics has been investigated by light scattering [3-6], turbidity [7-9], X-ray crystallography [10], and real space observation by confocal laser scanning microscopy [6,11]. In the fibrinogen-fibrin conversion, it has been reported that appreciable effects appear by the pH change [12], ionic strength [13], and divalent cations [14,15]. In addition, it has been found recently that various saccharides have unique effect [16]. Glucose and oligosaccharides retarded the fibrin gel formation dependently being less significant with the increases in the polymerization degree of glucopyranose units, and polysaccharides enhance with the molecular weight. Those novel characteristics are of great importance for the studies of blood coagulation and clinical aspects, and the detailed analysis of interaction mechanism is desired.

In the present paper, we report on the study of the stereoisomer effect of disaccharides, maltose, isomaltose, and cellobiose, on the fibrin gel formation by using light scattering combined with turbidity measurements. The similar effects by monosaccharides will be reported elsewhere. Furthermore, the release of fibrinopeptides by thrombin was examined by HPLC concerning the effect on enzymatic activity of thrombin by saccharides. NMR analyses were also used to examine the interaction of saccharides with fibrinogen.

2. EXPERIMENTAL

Bovine fibrinogen (clottability 97%, Sigma-Aldrich)

was dissolved in a phosphate buffer saline (PBS, pH 7.4), and this solution was dialyzed thoroughly against PBS to remove citric acid, chelating agent, and aggregates. Concentration of fibrinogen was determined by the absorbance at 280 nm using an extinction coefficient 1.51 ml/mg·cm. Bovine thrombin (Wako Pure Chemical) solution was also prepared in PBS. Special care was paid to avoid unnecessary adsorption to vessels. Fibrinogen solution was prepared to make a final concentration of 2.2 mg/ml, and the final thrombin concentration was 0.005 NIH units/ml in the whole measurements.

Disaccharides used were maltose (Wako Pure Chemicals), isomaltose and cellobiose (Sigma-Aldrich), and are of reagent grade. The final concentration was adjusted to 10 mg/ml. Figure 1 shows the molecular structure of disaccharides used in the experiments. All are glycoside-glucose types different with each other by the type of linkage.

Dynamic light scattering measurement as well as the scattered light intensity measurements were carried out using a homemade spectrophotometer and an ALV-5000E multiple-tau digital correlator to obtain the correlation function, and the details of the apparatus was described elsewhere [17]. Ar ion laser with the wavelength of 488.0 nm was used as a light source.

Turbidity measurements were performed using spectrophotometer (Hitachi U-2000A). Wavelength dependence of turbidity was obtained scanning over 400 to 800 nm at every 5 minutes. It should be noted that light scattering method is effective to analyze the process of protofibril formation to lateral aggregation and turbidity is sensitive to large aggregates in the process of lateral aggregation to gel formation.

For the dispersion of long rodlike particles, turbidity τ is related to the mass per length ratio μ and radius of rodlike particle (aggregates) as

$$C/\tau\lambda^3 = A/\mu + B(r^2/\mu)/\lambda^2 \qquad (1)$$

where λ is the wavelength of incident beam, A and B are the constant relating to the refractive index and the degree of its increment, and C the concentration. μ corresponds to the lateral aggregation number.

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 achieved 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. [18] with the following modifications: instead of ammonium acetate buffer, 0.1 % TFA or 0.1 % TFA in acetonitrile were used for

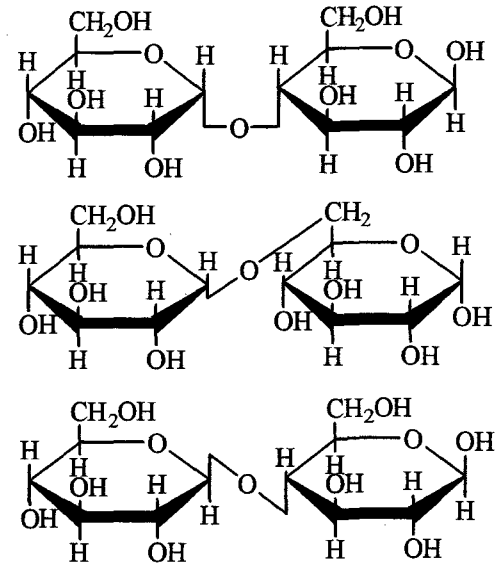

Fig. 1. Molecular structure of used disaccharides. top: maltose (α-1,4 linkage), middle: isomaltose (α-1,6 linkage), and bottom: cellobiose (β-1,4 linkage).

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 carried out at 37 °C ($\pm$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.

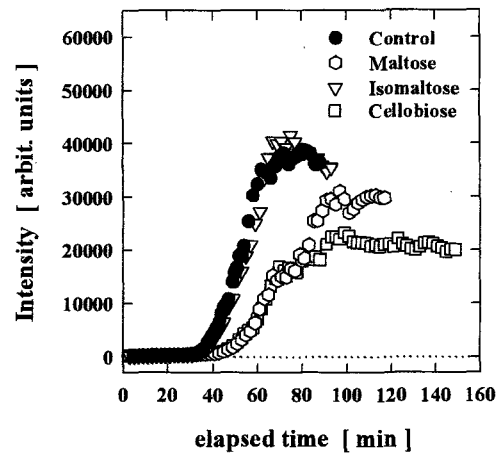

Fig. 2. Time evolution of the scattered light intensity at the scattering angle 30° for the fibrinogen-thrombin systems with various disaccharides. Control represents the fibrinogen -thrombin system without any disaccharides.

3. RESULTS AND DISCUSSION

Figure 2 shows effects of the addition of various disaccharides on the time evolution of scattered light intensity. The increases of them result from the aggregation of fibrin monomers, protofibril formation and growth of it, lateral aggregation of protofibrils, and network formation (gelation) in the course of reaction. Scattering angle was 30° and not zero. Although interference effect at finite scattering angle may cause marked decrease of scattered light intensity with the growth of long rod, rather smooth curves of time evolution were obtained because of the remaining unevenness of original fibrinogen sample and protofibrils. It has been confirmed previously for the fibrinogen-thrombin system that the rapid increase of scattered light intensity corresponds to the gelation point. That is, the gelation point of the control system locates at 40~50 minutes of the elapsed time. The successive increase is due to the further lateral aggregation to networking fibrin fibers and formation of heterogeneous network, spatial distribution of scarce and dense network. In the presence of disaccharides, all the samples gelled. However, maltose and cellobiose retarded the increase distinctly and the plateau level decreased remarkably. The plateau level of cellobiose is fairly less than that of maltose, and this fact suggests that the network (fibrin clot) in the presence of cellobiose is more homogeneous than that of maltose. In case of the addition of glucose [16], a little larger retardation effect has been observed, and the decay time distribution was very broad spanning down to the time corresponding to the motion of protofibrils. This fact means that the network is very flexible consisted of weak (thin) fibrin fibers (lateral protofibril aggregates). Therefore, the number of fibrin fibers composing the network is many (the number of networking points is large), and the formed network becomes homogeneous. The similar situation should occur in the cases of the addition of maltose and cellobiose. On the other hand, isomaltose has no effect and the time evolution is almost the same as the control.

Figure 3 depicts the results of turbidity analyses based on the equation (1). μ value for the control sample increases markedly around at 50 minutes indicating the gelation point in accordance with the results of light scattering. In contrast, the stepwise increase of μ values for the samples with maltose and cellobiose is retarded up to 70-80 min, and the plateau values seems to be much less than that of the control. Similarly, both the density and radius for the samples with maltose and cellobiose are fairly less than that of the control, respectively. Cellobiose is likely to have a larger effect in retarding the gelation and making the network homogeneous than maltose. However, the case of isomaltose no difference from the control was detected at all. Those characteristics are in good agreement with the results of light scattering.

The results of light scattering and turbidity analyses clearly reveal that the fibrin fibers com-

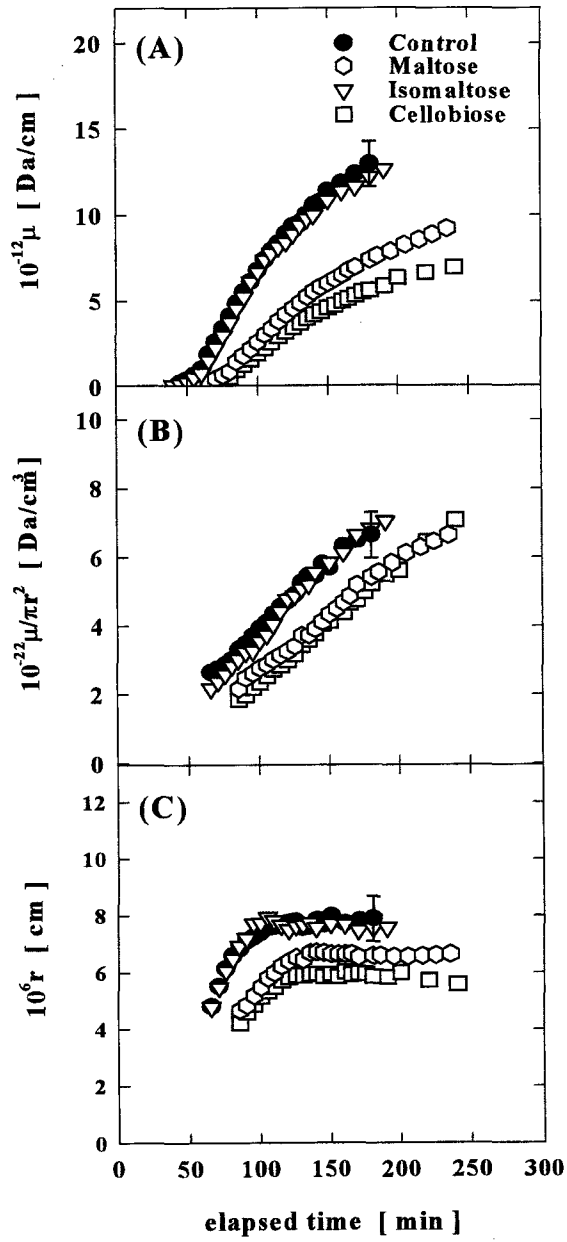

Fig. 3. Time variation of mass/length ratio μ (A), density $\mu/\pi r^2$ (B) and radius r (C) of fibrin fiber assembly. Meanings of the symbols are the same as those in Fig. 2. The bars in the figures correspond to $\pm$10% error.

posing the gel network in the presence of maltose and cellobiose are thin and loose ones. All the disaccharides examined here are the dimmer of glucose, and only the linking style is different from each other. That is, not only the molecular structure of monomer unit (data is not shown), but the linking style also is of great importance in affecting the fibrin gel formation. The steric structure causes the specificity.

According the HPLC analyses about FpA and FpB release, almost the same chromatograms were obtained for the samples with disaccharides as that of the control sample. the addition of disaccharides does not affect on the enzymatic activity of thrombin at all. Therefore, disaccharides must interact directly with fibrinogen/fibrin molecules maintaining the steric specificity. By using 400 MHz ^{1}H NMR, chemical shift spectra were measured for the mixture of fibrinogen and disaccharides. The resultant spectra were subtracted by the addition of respective spectra of fibrinogen and disaccharides to confirm whether the difference in spectrum intensity is observed. In all cases, distinct differences were observed in the NMR spectra. Disaccharides examined in the present study bind and interact directly with fibrinogen molecules. This fact is quite reasonable because it has been confirmed that glucose (dextran, too [16]) binds to fibrinogen directly.

The points at issue are the reason for no effect of isomaltose on the fibrin gel formation different from the cases of maltose and cellobiose, and at which conformation and position saccharides, including disaccharides, bind and/or interact with fibrinogen molecules. Isomaltose consists of α-1,6 linkage between two glucose units and has a warped conformation. Sterically specific interaction site may exist in fibrinogen molecule, and the isomaltose molecules bound to fibrinogen molecules might fail to function effectively, or do not bind to fibrinogen molecules effectively. More detailed analyses focusing to the site-specific interaction of fibrinogen are necessary, and such a study will bring useful information to the clinical applications concerning to the blood coagulation

4. CONCLUSION

Stereoisomer effects of glycoside-glucose type disaccharides, maltose, isomaltose, and cellobiose, on the fibrin gel formation process were investigated by using light scattering combined with turbidity measurements. Maltose and cellobiose retarded the fibrin gel formation, and the resultant fibrin fibers constituting the gel network were thin and loose ones. In contrast, isomaltose showed the same temporal growth as the control (without disaccharides), and the fibrin gel formation and the characteristics of fibrin fibers were not affected at all. Disaccharides do not affect on the enzymatic activity of thrombin and FpA and FpB release, but interact with fibrinogen molecules directly. The binding structure greatly affects the growth of fibrin fibers.

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