# The Effects of pH on the Generation of Turbidity and Elasticity Associated with Fibrinogen-Fibrin Conversion by Thrombin Are Remarkably Influenced by Sialic Acid in Fibrinogen

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**In fibrinogen-fibrin conversion by thrombin, the polymerization of a fibrin monomer is accompanied by gelation and an increase turbidity. Since sialic acids at the terminal of the carbohydrate chains bound to fibrinogen are part of the low affinity calcium binding site necessary for polymerization, they are closely involved in the network structure of fibrin clots. Fibrin clots derived from asialofibrinogen exhibited definite differences in turbidity and elasticity compared with those derived from intact fibrinogen, and were markedly dependent on the pH during the reaction. The turbidity during polymerization of fibrin, evaluated according to the absorbance at 350 nm, was maximum at pH 6.5—7.0, but it decreased in the other pH ranges, with the changes being unremarkable at higher pH levels but remarkable at lower pH ranges. The turbidity of fibrin derived from asialofibrinogen was far higher than that from intact fibrinogen near neutrality, but decreased rapidly and was lower than in intact fibrinogen at higher and lower pH ranges. Concerning the elasticity evaluated by thromboelastography, the coagulation time (*k*) and the maximum amplitude (*ma*) were lower in asialofibrinogen, indicating a deterioration of the clotting function of fibrinogen with the loss of sialic acid. These results suggest that sialic acid bound to fibrinogen is closely related to the fibrin network formation in blood coagulation, which is the most important function of fibrinogen, and plays a functional role in the stabilization of fibrin clot formation against environmental changes, including pH.**

**Key words** fibrinogen; asialofibrinogen; sialic acid; turbidity; elasticity; pH; fibrinogen-fibrin conversion

The final stage of blood coagulation reaction is the fibrinogen-fibrin conversion mediated by thrombin. This reaction is initiated by the release of fibrinopeptides by thrombin and is terminated with the formation of macromolecular polymers by the association of generated fibrin monomers and the production of stabilized fibrin bound by intermolecular covalent bonds.[1] Among these reactions, the release of fibrinopeptides and the formation of intermolecular covalent bonds, *i.e.* γ-glutamyl-(ε-lysine)–isopeptide bonds, by coagulation factor XIII are enzyme-mediated reactions, and their reaction mechanisms have been largely elucidated.[2] However, the association of fibrinmonomers, which is a non-enzyme reaction, and the subsequent formation of fibrin clots are the most drastic steps, accompanied by the appearance of turbidity and gelation, but their mechanisms remain largely unknown.

The carbohydrate chains in fibrinogen have been shown by thrombin or snake venom coagulation enzyme to have a considerable effect on fibrin clot formation.[3] We previously reported that sialic acids at the terminal end of the carbohydrate chain are deeply involved in the network formation of fibrin in the process of its polymerization and, thus, have a clear effect on the turbidity, rigidity, solubility and other properties of the fibrin gel at physiological pH.[4]

In this study, to further evaluate the role of sialic acid in fibrinogen-fibrin conversion, we studied the effects of pH on the development of turbidity and gelation in fibrin clot formation and observed that changes in the turbidity and those in the elasticity of fibrin gel, which are both influenced by pH, are not necessarily parallel.

## MATERIALS AND METHODS

**Preparation of Fibrinogen** 90% clottable fibrinogen from pooled human plasma (AB Kabi Vitrum, Stockholm, Sweden) was further purified. Namely, crude fibrinogen was dissolved at a concentration of 1% in 10 mM of a citrate buffer, pH 6.5, containing 0.15 M NaCl, and was allowed to stand overnight in a cold room (4 °C). Cold insoluble globulin that precipitated was removed by centrifugation, and while the supernatant was maintained at −3 °C, fibrinogen was precipitated by the glycine–ethanol method.[5] The wet precipitate was re-dissolved at 0.5% concentration with 55 mM citrate buffer, pH 6.4; 53.3% ethanol was added to this solution at concentration of 8%, and the fibrinogen precipitated was recovered by centrifugation. This fibrinogen, dissolved with 50 mM Tris–HCl, pH 7.5, containing 1 mM EDTA and 50 mM NaCl, was dialyzed against the same buffer, and was subjected to organomercurial-agarose (AFFI-GEL 501, Bio Rad Laboratories, Richmond, CA, U.S.A.) treatment by the method of McDonagh *et al.*[6] The fibrinogen, thus obtained, had a clottability of 97% or above.

**Preparation of Asialofibrinogen** Purified fibrinogen was dissolved at 0.5% concentration with 10 mM imidazole–HCl, pH 6.3, containing 0.15 M NaCl, then neuraminidase (EC 3.2.1.18, *Clostridium perfringens*, Worthington Biochemical Corporation, New Jersey, U.S.A.) was added at 5 mU/mg fibrinogen. The reaction mixture was incubated at 37 °C for 5 h. The resultant solution was dialyzed for 24 h against 0.15 M NaCl at 4 °C and lyophilized. This preparation of asialofibrinogen was hydrolyzed with diluted acid, and the sialic acid freed was assayed by HPLC using a TSKgel-SCX column (Tosoh, Tokyo, Japan)[7] or the thiobarbituric acid method,[8] which confirmed the

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nearly complete release of sialic acid. The clottability of this asialofibrinogen was retained at 96% or above.

**Evaluation of Changes in the Turbidity with Fibrin Clot Formation** Fibrinogen or asialofibrinogen was dissolved at a 0.5% concentration with buffers at various pH levels containing 5 mM $Ca^{2+}$ and 0.15 M NaCl, then 2.5 ml of each solution was placed in a cell, 0.5 ml (5 U) of thrombin (500 U/vial, Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) in the same buffer was added, and the absorbance at 350 nm was immediately recorded after the addition of thrombin with a spectrophotometer (U-3200, Hitachi, Ltd., Tokyo, Japan).

**Evaluation of Changes in the Elasticity by Thromboelastography** Fibrinogen was dissolved at 1% concentration with buffers at various pH levels; 300 $\mu$l of each solution was placed in a reaction cup equipped in a thromboelastograph (Clot-tracer, model TE-400, Erma, Inc., Tokyo, Japan), and each was incubated with 50 $\mu$l thrombin (2 U) at 37 °C. Experimental procedures on thromboelastography were based on the method of Hartert.[9]

The buffers used in this study were as follows.

pH 5.0—7.0: sodium acetate–NaOH
pH 6.0—8.5: imidazole–HCl
pH 7.5—9.0: Tris–HCl
pH 8.5—10.0: Taps–HCl

All buffers contained 5 mM $Ca^{2+}$ and 0.15 M NaCl. In consideration of the effects of the composition of the buffers, data obtained using two buffers with the same pH prepared in different compositions were compared and corrected.

**Determination of Protein Concentration** Fibrinogen concentration was determined by the absorbance at 280 nm of the fibrinogen solution dissolved in 0.2 N NaOH–40% urea, using $A_{280}=15.4$ for 1% fibrinogen.[10]

## RESULTS

**Sialic Acid in Asialofibrinogen Preparation and Its Clottability** It is important that the clotting activity of asialofibrinogen does not decrease during neuraminidase treatment for the release of sialic acid from fibrinogen. Therefore, fibrinogen was allowed to react with various amounts of neuraminidase, and the relationship between the amount of sialic acid released and the clottability of asialofibrinogen was investigated. As shown in Fig. 1, after 5 h of reaction, asialofibrinogen treated with 5 mU of neuraminidase retained 96% clottability, and the sialic acid in fibrinogen was almost freed. The removal of sialic acid by treatment with 1 mU of neuraminidase was not sufficiently achieved in 5 or more hours of the reaction. When 50 mU of neuraminidase was used, sialic acid was not detectable in the preparation, but clottability was considerably decreased. From the results of SDS–PAGE analysis, the decrease of clottability is possibly based upon the degradation of the A$\alpha$-chain, which is sensitive against proteases presumably mixed in the authentic sample of neuraminidase.[11]

**Effects of pH on the Turbidity** Fibrinogen and asialofibrinogen were treated with a fixed amount of thrombin

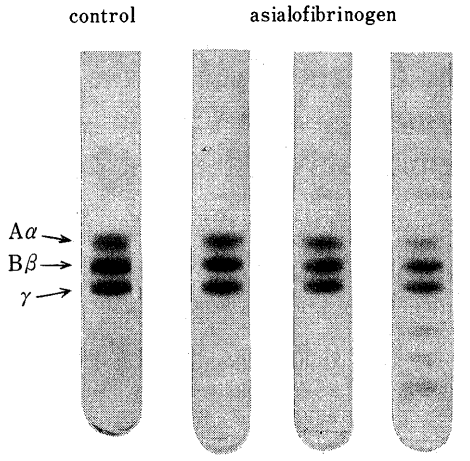


Fig. 1. The Clottability and Residual Sialic Acid in Asialofibrinogen, and SDS-PAGE Analysis of Its Preparation

A time course analysis was examined prior to the preparation of asialofibrinogen and the reaction with neuraminidase was carried out for 5 h. The amount of sialic acid in fibrinogen was calculated from the data in ref. 7 (6.46 mol sialic acid/mol fibrinogen, and was represented by mol/mol (asialo)fibrinogen).

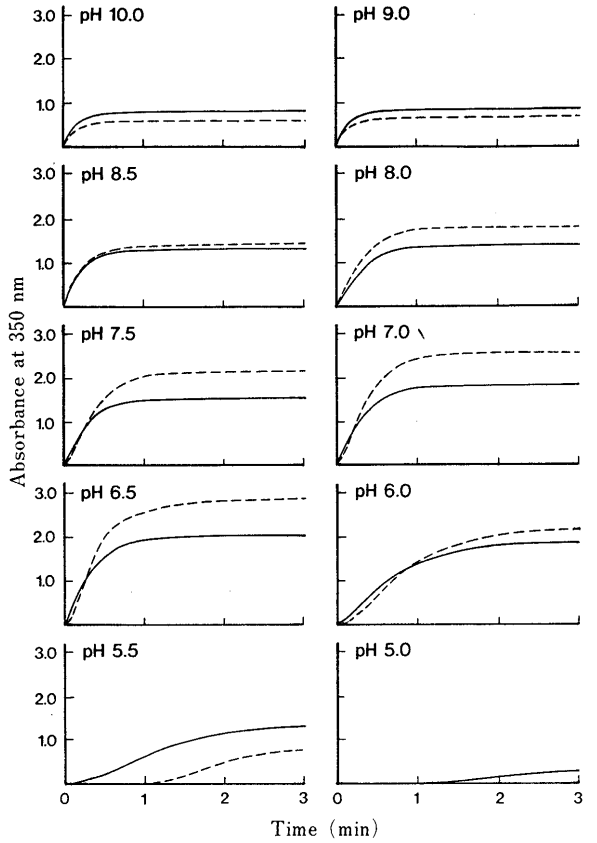


Fig. 2. Time Course Change in Turbidity Accompanying Fibrin Polymerization at Various pH Values

Fibrinogen or asialofibrinogen was dissolved in each buffer solution with respective pH values at a concentration of about 1%, and then diluted at a 0.5% with an appropriate volume of the same buffer calculated from the values of the absorbance at 280 nm of the initial fibrinogen solution. Absorbance at 350 nm was measured with a Hitachi Spectrophotometer U-3200 at room temperature. ——, fibrinogen; ------, asialofibrinogen.

in buffers with varying pH, and the time course of the changes in turbidity was examined according to the absorbance at 350 nm (Fig. 2). The turbidity was reported

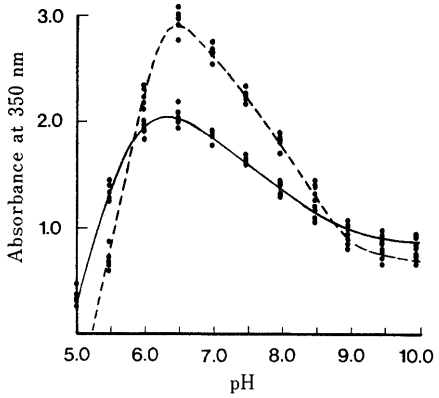


Fig. 3. The Relationship between pH and Maximum Turbidity

Maximum turbidity at respective pH values was determined as the absorbance at 350 nm after 5 min reaction with thrombin. ——, fibrinogen; ------, asialofibrinogen.

earlier to be higher for asialofibrin than that of intact fibrin at physiological pH levels.[4] In this study, the turbidity of asialofibrin was higher than intact fibrin below pH 8—8.5, the pattern of development of turbidity was not different between asialofibrin and intact fibrin at pH 8.5, and the turbidity of asialofibrin became lower than that of intact fibrin at a higher pH. Also, the turbidity was the highest around pH 6.5 for both fibrins, then decreased rapidly at lower pH levels. The turbidity of asialofibrin was lower than that of intact fibrin at pH 5.0 ($A_{350}=0.082$, 30 min), but no increase in $A_{350}$ was observed in asialofibrin, even when the measurement was prolonged by 30 min. Therefore, the turbidity of both fibrins at pH 8.5—10 was far below the turbidity at the neutral pH region, and the effect of pH disappeared in both fibrins at a pH of 9—10, but the changes in turbidity were remarkable at pH 6.5—5.

Figure 3 shows the relationship between pH and the maximum turbidity. The turbidity of asialofibrin was higher between pH 8.7 and 6.1, but this was reversed at pH levels higher or lower than this range. Moreover, concerning the time from the addition of thrombin to the development of the turbidity, both fibrins developed turbidity immediately after the addition of thrombin above pH 7, but the time of turbidity onset was prolonged with decreasing pH. The retardation of the development of turbidity at lower pH was especially notable in asialofibrin, suggesting that sialic acid had a greater effect on the fibrin network formation at the low pH region.

**Effects of pH on the Elasticity** Changes in elasticity associated with the polymerization of fibrin and asialofibrin were examined serially at various pH levels by thromboelastography, and the role of intramolecular sialic acid in the gelation of fibrin molecules was evaluated. As shown in Fig. 4, in intact fibrinogen, the thromboelastogram showed little change above pH 7, and the resultant fibrin had sufficient elasticity in this pH range. This is also clear in the values of the thromboelastographic parameters shown in Table I. The reaction time ($r$) was 3.5—3.8 min, the coagulation time ($k$) was 1.2 min, and the maximum amplitude ($ma$) was 47—51 mm; the elasticity appears to be unaffected by pH in this pH range. However, at pH 6.5, the elasticity began to decrease in the thromboelastogram, and clear changes were observed in the values of

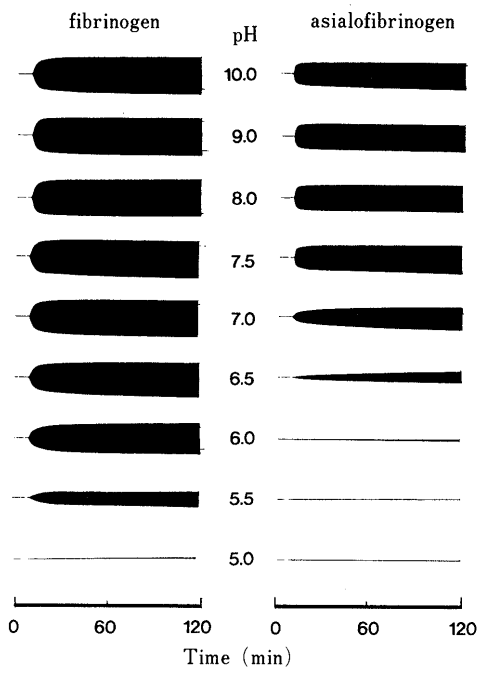


Fig. 4. Continuous Change in Elasticity Associated with the Formation of Fibrin and Asialofibrin Clot by Thrombin on Thromboelastography

The test fibrinogen solution was preincubated in a reaction cup equipped with a thromboelastograph (Clot-tracer, model TE-400, Erma Inc., Tokyo, Japan) at 37°C for 3 min, and then the reaction was started by the addition of thrombin. Details are in the previous paper.[4]

TABLE I. Thromboelastographic Parameters of Fibrinogen and Asialofibrinogen

| pH | Fibrinogen | | | Asialofibrinogen | | |
|---|---|---|---|---|---|---|
| | $r$ (min) | $k$ (min) | $ma$ (mm) | $r$ (min) | $k$ (min) | $ma$ (mm) |
| 10.0 | 3.6 | 1.2 | 50 | 3.6 | 1.2 | 37 |
| 9.5 | 3.7 | 1.2 | 49 | 3.7 | 1.2 | 36 |
| 9.0 | 3.6 | 1.2 | 48 | 3.7 | 1.2 | 37 |
| 8.5 | 3.7 | 1.2 | 50 | 3.5 | 1.2 | 38 |
| 8.0 | 3.5 | 1.2 | 49 | 3.7 | 1.2 | 36 |
| 7.5 | 3.7 | 1.2 | 48 | 3.8 | 1.4 | 38 |
| 7.0 | 3.8 | 1.4 | 51 | 3.8 | 4.0 | 32 |
| 6.5 | 4.2 | 2.1 | 40 | 5.6 | — | 16 |
| 6.0 | 4.7 | 27.7 | 24 | 12.2 | — | 3 |
| 5.5 | 5.4 | 57.4 | 21 | — | — | — |
| 5.0 | 7.5 | — | — | — | — | — |

Reaction time ($r$); time (min) from the initiation of reaction to the amplitude of 1 mm on the thromboelastogram. Coagulation velocity ($k$); time (min) to reach to 2 cm from 1 mm of amplitude. Maximum amplitude ($ma$); distance of amplitude (mm) at the time when the maximum amplitude or equilibrium of reaction is attained. The values represent the mean of three experiments.

the parameter. Furthermore, at pH 6.0, the value of $k$ increased rapidly, and $ma$ decreased to about 50% of that at pH 7.0. At pH 5.5, these changes became more notable (data not shown), and at pH 5.0, few changes were observed in the parameters, although low fluctuations were seen in thromboelastogram near the end of the 2 h measurement period. In asialofibrin, the thromboelastogram showed nearly the same pattern above pH 7.5, indicating a fixed elasticity, and asialofibrin was considered to retain its clotting ability. The values of $r$, $k$ and $ma$ were 3.6 min, 1.2 min and 37 mm, respectively, showing little difference at the high pH range. However, unlike

intact fibrin, a reduction of elasticity of asialofibrin began at pH 7.0, and clear changes were observed in *k* and *ma*. At pH 6.5, *ma* was 16 mm, which is lower than 50% of the value at pH 7.5, and *k* was unmeasurable. At pH 5.5 or below, no changes suggestive of gel formation were noted in the thromboelastogram or the values of the parameters. When the above results are compared between fibrin and asialofibrin, no difference was observed in the time required for the development of elasticity between the two fibrins at pH 7.0 or higher. However, differences in the values of the parameters began to appear at lower pH levels, and the deterioration of elasticity was more notable in asialofibrin. Especially, the *ma* was markedly lower in asialofibrin than in intact fibrin over the entire pH range examined, suggesting that sialic acid in fibrinogen affects the development of elasticity, which is the most important property of fibrinogen.

## DISCUSSION

In the blood clotting reaction as a protective mechanism of the body, the formation of thrombi with sufficient strength is necessary to prevent the loss of blood from injured vessels. For this purpose, fibrin clots formed in this process must have appropriate elasticity and/or rigidity.

The conversion of fibrin monomers to a fibrin polymer, which results from the release of two kinds of fibrinopeptide from the N-terminal region of fibrinogen by thrombin, is prevented in 1 M NaBr solution so that the fibrin monomer is present as a transparent solution.[12] However, the polymerization reaction begins immediately by dilution with media such as a neutral buffer containing calcium, and turbidity appears with the progression of gelation. These observations suggest a close relationship between the development of turbidity and gelation in the process of fibrinogen-fibrin conversion. Changes in turbidity associated with fibrin clot formation are reportedly affected by phosphoric acid residues binding with fibrinogen molecules[13] and carbohydrate chains,[14] and especially by the presence or absence of sialic acid residues at their terminal end.[15] The binding state of these attached molecules are suggested to influence the clotting time, solubility, rigidity and protease sensitivity of fibrin clots.[4]

Previously, we reported that the turbidity associated with fibrin polymerization is increased but its elasticity is reduced when fibrinogen lacks sialic acid residue.[4] Evaluation of the changes in the relationship between these two contradicting phenomena according to reaction conditions, including pH, is considered to provide important information for clarifying the mechanism of fibrin clot formation.

In this study, maximum turbidity was found around at pH 6.5, the turbidity decreased gradually at a high pH range, and the decreasing pattern of turbidity showed no marked changes above pH 9. However, below pH 6, the turbidity changed remarkably, and the network structure formed under this condition may be different from that formed at higher pH levels. These phenomena were more distinct in asialofibrin than in intact fibrin, and a rapid loss of turbidity was confirmed, especially in a low pH range. The elasticity of fibrin clots was markedly affected at low pH levels, but little effect was observed above physiological pH. The elasticity decreased at low pH in both fibrins, but the change was more notable in asialofibrin. Moreover, the elasticity of the fibrin gel derived from asialofibrinogen was lower than that of fibrin gel derived from intact fibrinogen for all pHs examined. Furthermore, at a high pH range, sufficient elasticity remained in spite of low turbidity. It is therefore ascertained that the development of turbidity and elasticity in the fibrinogen-fibrin conversion did not necessarily proceed in parallel. These findings suggest that the environment for the polymerization of fibrin monomer molecules, as well as the presence or absence of highly polar residues such as sialic acid, are important factors in fibrin network formation.

The development of turbidity accompanying the polymerization of a fibrin monomer is based on the network formation of fibrin fibers by means of association, which involves either side-to-side or end-to-end noncovalent intermolecular binding from a protofibryl state.[16] If the formation of this network structure is dependent on various bonds that make up the common high-order structures of protein, namely the hydrophobic bond, hydrogen bond, electrostatic repulsion or attraction,[17] the pH of the reaction environment is considered likely to have a strong effect on these binding mechanisms.

Calcium is essential for fibrin polymerization, because the clotting ability of fibrinogen is greatly reduced in the presence of calcium chelating agents such as EDTA.[18] It has been reported that a slow rate of fibrin polymerization favours a high degree of lateral aggregation and thus the formation of thick fiber bundle, and that sialic acid residues contribute to electrostatic repulsion, which appears to decrease the lateral aggregation of fibrin protofibryl, resulting in thinner fiber bundles than those of asialofibrin.[19] From findings in this study, changes in turbidity between fibrin and asialofibrin during their polymerization process are considered to be based on the functions of sialic acid, *i.e.* influences to the lateral aggregation rate and the state of the fibrin fiber bundle described above. Also, low elasticity in asialofibrin is assumed to occur because sialic acid is a part of the low affinity calcium binding site, and asialofibrin to which is bound an insufficient amount of calcium does not possess the ability to form a stable fibrin clot, including the reaction by coagulation factor XIII.[20] It is speculated that such a molecular condition which affects the binding mechanism of the fibrin molecule in the association of the fibrin monomer is enhanced at a low pH and reflected in the turbidity and elasticity of the fibrin gel.

It has been known that there are fibrinogen molecules with normal sialic acid binding and those with abnormal sialic acid binding among fibrinogen molecules obtained from patients with some liver diseases and fibrinogen molecules with genetic defects, and that the clotting time of such abnormal molecules is prolonged.[21] These functional abnormalities of fibrinogen are considered to reflect the state of sialic acid in the fibrinogen molecule and to be the result of changes in the turbidity and elasticity

observed in this study.

Many plasma sialo-glycoproteins are metabolized during circulation and lose sialic acid, and as a result, galactose is exposed at the terminal of the carbohydrate chain. Thus, these glycoproteins are captured by receptors that recognized galactose, are degraded by reticuloendothelial cells, and their lives end.[22] The primary function of fibrinogen is to immediately form fibrin clots in the emergency of vascular injury, to repair the injury site, and thus, to prevent the loss of blood. Sialic acid in fibrinogen bears a functional role in forming stable fibrin clots, even when the pH of the body fluid is changed.

It is a reasonable mechanism for maintenance of the living body that fibrinogen, weakened of its intrinsic function by losing sialic acid, is eliminated from the circulation.

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