Biol. Pharm. Bull. **16**(5) 448—452 (1993) Vol. 16, No. 5

Sialic Acid in Fibrinogen: Effects of Sialic Acid on Fibrinogen–Fibrin Conversion by Thrombin and Properties of Asialofibrin Clot

Masayoshi OKUDE,* Aiko YAMANAKA, Yumiko MORIMOTO and Sumiyuki AKIHAMA

The First Department of Biochemistry, Meiji College of Pharmacy, 1–35–23, Nozawa, Setagaya-ku, Tokyo 154, Japan.

Received September 21, 1992

The final stage in a series of blood coagulating reactions is fibrinogen–fibrin conversion by thrombin. This reaction consists of fibrinopeptide A and fibrinopeptide B release, polymerization of fibrin monomer, and stabilized fibrin formation by factor XIII. The latter two reactions require calcium. In the present study there was no difference in the rate of thrombin-induced fibrinopeptide release between fibrinogen and asialofibrinogen where sialic acid in the terminal end of carbohydrate moiety of fibrinogen was removed by neuraminidase, but turbidity associated with asialofibrin clot formation was increased more rapidly. In asialo-derivatives, the dissolution time of the clots in high concentrated urea solution tended to be shortened and rigidity as a gel tended to be decreased. In measurement by thromboelastography there was no difference in the reaction time (*r*) between fibrinogen and asialofibrinogen, but the maximum amplitude (*ma*) was obviously decreased in asialofibrinogen. Furthermore, when the rate of cross-link formation between γ chains by F-XIII was compared, the production of γ-dimer in the same reaction time was found to be lower and formation of stabilized fibrin tended to be retarded in asialofibrinogen. Sialic acid in fibrinogen thus may clearly influence the polymerization of fibrin-monomer and the formation of cross-linked fibrin in a series of reactions for fibrinogen–fibrin conversion. This may be consistent with the theory that fibrinogen sialic acid residues are low affinity calcium-binding sites and influence fibrin assembly.

Keywords fibrinogen; asialofibrinogen; sialic acid; polymerization; stabilized fibrin; fibrinogen–fibrin conversion

Introduction

Fibrinogen is a plasma glycoprotein with a molecular weight of 340 kDa which plays the most important role, that is, clot formation, in a series of blood coagulating reactions. Its conversion to a final product of stabilized fibrin is considered to be divided into four stages as described below.[1)]

1. Limited hydrolysis of fibrinogen by thrombin, that is, release of firbinopeptide A (FP-A) and B (FP-B), and formation of fibrin monomer.[2)] 2. Formation of protofibryl by the association of fibrin monomer.[3)] 3. Formation of fibrin fibers by the association of protofibryl.[4)] 4. Cross-link formation by the blood coagulating factor XIII (fibrin stabilizing factor, F-XIII).[5)]

The mechanism involved in the appearance of turbidity associated with polymerization of fibrin or gel formation (increased elasticity and/or rigidity) in this series of reactions for fibrinogen–fibrin conversion, has not yet been adequately elucidated. Further, it has been suggested, but not demonstrated in detail, that the carbohydrate chain which is bound to Bβ and γ chain of fibrinogen molecules is closely related to the network formation in fibrin clots.[6)]

It has been demonstrated *in vitro* that asialofibrinogen obtained by removing sialic acid bound to the terminal end of fibrinogen carbohydrate moiety is different from intact fibrinogen in thrombin-induced clotting time.[7)] It has also been reported that there is no difference in coagulation parameters between fibrin and asialofibrin, and that the sialic acid residues do not influence fibrinogen–fibrin conversion.[8)] Thereafter, however, it was demonstrated that the turbidity of clots is higher for asialofibrin,[9)] and more recently, asialofibrin was described as being different from intact fibrin in the shape of fibrin fibers and interactions with calcium.[10)] On the other hand, there are some reports that more sialic acid is bound in fibrinogen obtained from patients with certain hepatic disorders[11)] and so-called abnormal fibrinogen with genetic molecular abnormality than in normal fibrinogen, and the clotting time induced by thrombin and snake venom coagulation enzyme tends to be increased commonly in such kinds of fibrinogen.[12)] Quite recently, an abnormal fibrinogen bound sialic acid-rich-carbohydrate to Aα chain was found, and abnormalities of its clotting property were revealed.[13)] These facts strongly suggest the possibility that sialic acid in fibrinogen functionally acts on the polymerization of fibrin and the mechanism of clot gel formation during hemorrhage.[14)] However, no report has been presented concerning the effect of sialic acid throughout the process of the fibrinogen–fibrin conversion, that is, the formation of fibrin clots, fibrin polymerization and cross-linking between fibrin molecules by F-XIII.

In the present study sialic acid was enzymatically removed from fibrinogen without damaging its coagulating function and the sialic acid free fibrinogen, namely asialofibrinogen was investigated for time-related changes in FP-A and FP-B release, polymerization, and stabilized fibrin formation, in comparison with intact fibrinogen; the role of sialic acid in fibrinogen–fibrin conversion was also evaluated.

Materials and Methods

Preparation of Fibrinogen Crude human fibrinogen (grade L, 90% clottable) obtained from AB Kabi Vitrum (Stockholm, Sweden) was further purified by ethanol precipitation according to the modified method of Doolittle *et al.*[15)] with the additional procedure of removing cold precipitated protein by allowing the fibrinogen solution to stand in a cold room. Contaminating F-XIII in the fibrinogen preparation was removed using organomercurial-agarose (Affi-gel 501, Bio-Rad Laboratories, Richmond, CA, U.S.A.) as reported by McDonagh *et al.*[16)] When this authentic sample was allowed to react with thrombin for a sufficient time in 0.01 M imidazole–HCl, pH 7.2 containing 0.15 M NaCl (abbreviated as imidazole buffer, unless otherwise specified) and analyzed using sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE),[17)] no γ-dimer or α-polymer was detected. In the control group (not treated with organomercurial agarose) γ and α monomers were converted to dimers and polymers, respectively. The purity of fibrinogen preparation was

judged from the result of thrombin-induced clottability and SDS-PAGE. The fibrinogen concentration was determined by measurement of the absorbance at 280 nm using alkaline-40% urea as solvent. The factor $A_{280}=15.4$ for a 1% (w/v) solution of fibrinogen was used in all calculations.[15] The clottability of this fibrinogen preparation was 96% or higher.

Preparation of Asialofibrinogen Release of sialic acid from fibrinogen by neuraminidase (EC 3.2.1.18, *Clostridium perfringens*, Worthington Biochemical Corporation, New Jersey, U.S.A.) was achieved by incubating a fibrinogen solution (5 mg/ml) in 0.01 M of imidazole–HCl, pH 6.3, containing 0.15 M NaCl with neuraminidase (0.005 U/mg of fibrinogen) at 37 °C for 5 h. The resultant solution was then dialyzed against 2 l of 0.15 M NaCl three times over a 1 d period and lyophilized.

Analysis of Sialic Acid Fibrinogen-bound sialic acid was freed from 10 mg fibrinogen by incubation in 1 ml of 0.1 N sulfuric acid for 1 h at 80 °C in an atmosphere of nitrogen. The hydrolysate was cooled for 3 min in an ice-water bath and then centrifuged with ultrafiltration, Ultracent-30 purchased from Tosoh (Tokyo, Japan), at 3500 rpm for 30 min in a cold room. Authentic *N*-acetylneuraminic acid was treated in parallel under the same conditions in order to correct the data obtained from the samples. Sialic acid in 10 μl of the filtrate was analyzed by cation exchange column chromatography on a Tosoh HPLC system (Tosoh, Tokyo, Japan) equipped with a CCDP pump, a UV-8000 detector, a Rheodyne 7125 sample injector valve (100 μl loop), and a strong cation-exchange polymer column, TSKgel-SCX (300 × 7.5 mm). Analysis was performed by isocratical elution using 1% of *ortho*-phosphoric acid as a mobile phase at a flow rate of 0.8 ml per minute at an ambient temperature (*ca.* 25 °C). The column effluent was monitored by a UV detector at 212 nm and the peak area was calculated by an integrator, Sic chromatocorder-11.[18]

Analysis of FP-A and FP-B After 10 U of thrombin was allowed to act on 1 ml of imidazole buffer solution of 1% fibrinogen or asialofibrinogen for an appropriate time, the reaction was terminated by heating for 3 min in a boiling water bath. After cooling, it was ultrafiltrated using Ultracent 30. FP-A and FP-B contained in the filtrate were determined on HPLC according to the method of Ruf *et al.*[19] Purified FP-A and FP-B for preparation of the calibration curve were purchased from Sigma Chemical Company (St. Louis, U.S.A.).

Measurement of Solubility of Fibrin Clots in 10 M Urea After 100 μl of imidazole buffer solution containing 1% fibrinogen was mixed with 10 μl of F-XIII (0.5 U), 90 μl of thrombin (1 U) dissolved in imidazole buffer containing 0.005 M Ca^{2+} was added and the mixture was allowed to react at room temperature for various periods. The reaction was terminated by heating for 3 min in a boiling water bath. The clot was suspended in 10 M urea containing 2-mercaptoethanol (0.1%) and shaken continuously at a constant rate in a thermostat maintained at 40 °C; time to the dissolution and disappearance of the clot was measured. 0.001% bromophenol blue was added to facilitate dissolution of the clot in the urea solution.

The purified F-XIII from blood plasma was the kind gift of Dr. T. Suzuki of the authers' college. The amount of F-XIII to completely convert γ and α of fibrinogen (1 mg) to γ-dimer and α-polymer after 6 h of reaction in the presence of calcium was defined as one unit by the dilution method.

Measurement of Turbidity Associated with Fibrin Clot Formation After 2.5 ml of 1% fibrinogen solution in imidazole buffer containing 0.005 M Ca^{2+} and 0.5 ml of F-XIII (5 U) were put in a cell, 0.5 ml of thrombin (25 U) was added and the change of absorbance at 350 nm was measured immediately.

Hardness Test of Stabilized Fibrin To 4 ml of 1% or 0.5% fibrinogen in imidazole buffer, 100 μl of F-XIII (20 U) and 100 μl of an appropriate concentration of thrombin were mixed and allowed to react overnight at room temperature in a glass cup (2 cm in diameter). In the measurement of rigidity a plastic disc (diameter: 1.5 cm) was placed on a clot gel and the gel was pressed with constant force using a hardness tester (Model KM, Kiya Seisakusyo Ltd., Tokyo, Japan). The value of pressure required to bury the disc in the gel was recorded.

Measurement of Fibrin Clot Formation Pattern by Thromboelastography A thromboelastograph (Clot-tracer, model TE-400, Erma Inc., Tokyo, Japan) equipped with a four-channel reaction cup and a recorder was used. To 200 μl of 1% fibrinogen sample dissolved in imidazole buffer containing 0.005 M Ca^{2+} in a reaction cup, a solution prepared previously by incubating 100 μl of F-XIII (0.5 U) and 50 μl of thrombin (2 U) at 37 °C for 10 min was added and measurement was started immediately. As a control, imidazole buffer was used in place of F-XIII. Thromboelastograms were analyzed by the method of Hartert[20] as described below.

reaction time (*r*): time (min) from the initiation of reaction to the amplitude of 1 mm on the chromatogram.

maximum amplitude (*ma*): distance of amplitude (mm) at the time when the maximum amplitude or equilibrium of reaction is attained.

Measurement of the Extension of Cross-Linked γ-Chain (γ-Dimer) Formation by SDS-PAGE and Densitometer To 100 μl of 1% fibrinogen in imidazole buffer containing 0.005 M Ca^{2+}, 10 μl of F-XIII (1 U) and 10 μl of thrombin (1 U) were added and the mixture was allowed to react at 30 °C for appropriate periods. The reaction product was dissolved in the sample buffer containing 1% SDS, 0.1% 2-mercaptoethanol, 0.01% bromophenol blue and 10 M urea, and 20 μl of this solution was subjected to SDS-PAGE.[17] After electrophoresis, separated proteins in gel were scanned on a densitometer (Densitron model-PAN, Jookoo Co., Ltd., Tokyo, Japan).

Results and Discussion

In the preparation of asialofibrinogen it is important to remove sialic acid without decreasing the coagulation activity which is a function of fibrinogen. Therefore, fibrinogen was allowed to react with various amounts of neuraminidase and the relationship between the amount of sialic acid released and coagulation activity was investigated. As shown in Fig. 1, 96% or more of sialic acid was released after about 3 h of reaction between 1 mg of fibrinogen and 0.05 U of neuraminidase; however, the coagulation activity of asialofibrinogen obtained by this reaction was decreased to 85 to 90%. When 0.005 U of neuraminidase was used, 97% or more of sialic acid was removed after 5 h of reaction and 95% or more of coagulation activity was retained. When 0.001 U of neuraminidase was used, about 90% of sialic acid was hydrolyzed after ten hours of reaction, but the coagulation activity of asialofibrinogen obtained by this reaction was decreased to 80% or less. It has been demonstrated that the Aα chain of fibrinogen shows the highest sensitivity to proteases.[21] It was concluded from the results of SDS-PAGE analysis in the three kinds of asialofibrinogen described above that coagulation activity was decreased by such a series of neuraminidase treatment, because the Aα chain of fibrinogen was damaged by a trace of proteases which were presumably mixed in the authentic sample of neuraminidase (data not shown). Based on these results, sialic acid free fibrinogen with a sufficient coagulation function

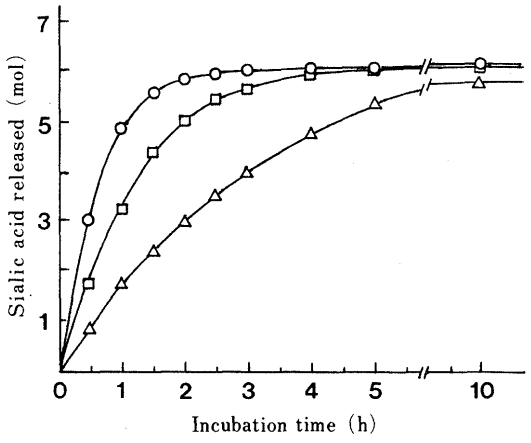

Fig. 1. Time Course of Sialic Acid Release from Fibrinogen by Treatment of Various Amounts of Neuraminidase

The amounts of neuraminidase per mg of fibrinogen used were: 0.05 U (○), 0.005 U (□) and 0.001 U (△). Analysis of residual sialic acid in fibrinogen after treatment by neuraminidase was done on HPLC after hydrolysis with mild acid. The amount of sialic acid released from fibrinogen was calculated from the data in ref. 18 (6.46 mol sialic acid/mol fibrinogen). Details are described in Materials and Methods.

was obtained by reaction with 0.005 U of neuraminidase and 1 mg of fibrinogen for 5 h at 37 °C.

Fibrinogen–fibrin conversion is commenced by the limited hydrolysis of Arg-14 in the Aα chain of fibrinogen followed by that of Arg-16 of the Bβ chain to release two kinds of peptides, FP-A and FP-B.[22] Therefore, for purposes of investigating the role of sialic acid in the mechanism of fibrinopeptide release, thrombin was allowed to act on fibrinogen and asialofibrinogen under the same conditions and the release rates of the two kinds of fibrinopeptide were measured and compared. As shown in Fig. 2, no difference was noted in the release pattern of FP-A and FP-B between the two types of fibrinogen, indicating that sialic acid elicits no change, at least in the action site of thrombin on fibrinogen molecules. Accordingly, the role of sialic acid in fibrinogen–fibrin conversion may be fulfilled after this stage of reaction.

It has been considered that fibrin-monomer resulting from the release of the two kinds of fibrinopeptide performs continuous non-enzymatic side to side and then end to end association between fibrin molecules to form fibrin-polymer.[23] Furthermore, as the site of binding is approached, cross-links of ε-(γ-glutamyl)-lysine are formed by the action of F-XIII$_a$ between α chains and between γ chains, respectively, and the reaction is terminated by the formation of rigid stabilized-fibrin.[24] Fibrin-polymer as an intermediate of these reactions is readily soluble in 1% monochloroacetic acid or higher concentrations of an aqueous solution of urea. Since the stabilized-fibrin receiving the action of F-XIII is only slightly soluble in these solutions, however, its solubility is used to measure the activity of F-XIII.[25] Accordingly, the influence of sialic acid on fibrin-polymer and stabilized fibrin formation was assessed by comparing the dissolution rates of fibrin and asialofibrin clots which differed in reaction time with enzyme, in 10 M urea. The result in Fig. 3 shows that both fibrin clots were dissolved within about 5 min in the absence of F-XIII and there was no notable significant difference between the two substances. This was presumably because their clots were in the fibrin-polymer state covalent bonds were not formed by F-XIII, and therefore, these were brought back to fibrin-monomer as a result of urea-induced dissociation and readily dissolved. In the presence of F-XIII, however, the dissolution time of clots was increased together with reaction time with thrombin and results reflecting the formation of cross-links by F-XIII were obtained. Asialofibrin clots tended to be dissolved more rapidly than fibrin clots in each reaction period. The difference in dissolution time after the same time of reaction between the two types of fibrin clots was considered to reflect the difference in the amounts of stabilized fibrin

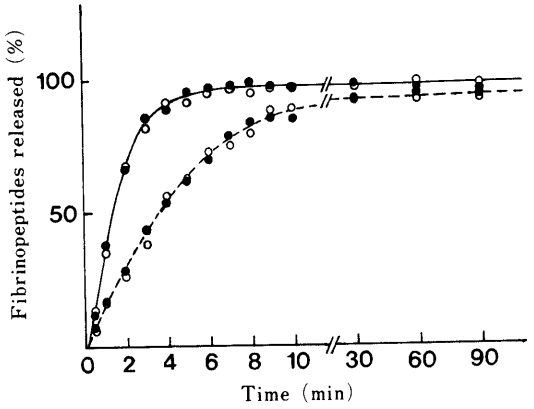

Fig. 2. Time Course of FP-A and FP-B Release from Fibrinogen and Asialofibrinogen

Values of FP-A and FP-B released from fibrinogen samples were expressed as the percentages for theoretical amount of them. ●, fibrinogen; ○, asialofibrinogen; ——, FP-A; – – –, FP-B.

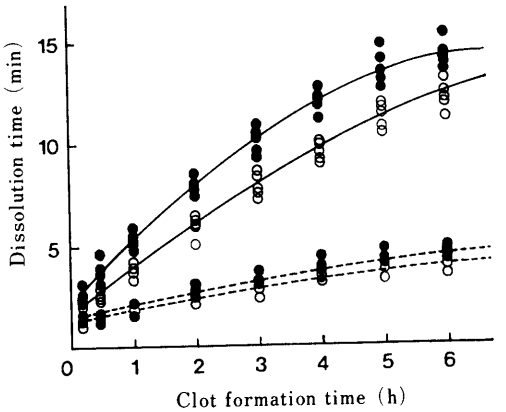

Fig. 3. Dissolution Time of Fibrin and Asialofibrin Clots in 10 M Urea

Fibrinogen (●) or asialofibrinogen (○) solution and thrombin were mixed and reacted at room temperature for 0, 5, 20, 60, 120, 180, 240, 300 and 360 min in the absence (– – –) or presence (——) of F-XIII. Measurement of dissolution time of clots formed in each reaction period was carried out on five specimens.

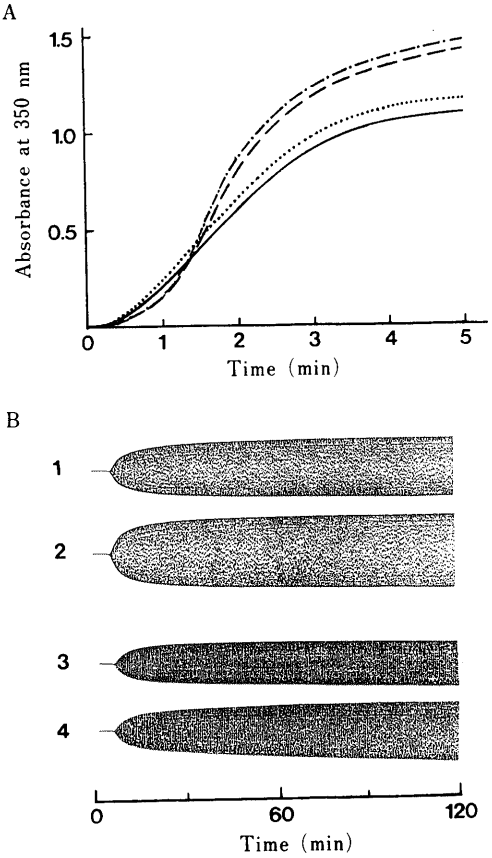

Fig. 4. Turbidity Change (A) and Clot Formation Pattern on Thromboelastograph (B) of Fibrin and Asialofibrin

Continuous change of turbidity associated with fibrin and asialofibrin by thrombin was analyzed by measuring of absorbance at 350 nm for 5 min. The reactions were performed in the absence (——, fibrin; – – –, asialofibrin) or presence (------, fibrin; -·-·-, asialofibrin) of F-XIII. Clot formation was analyzed by thromboelastography on fibrinogen (1, 2) and asialofibrinogen (3, 4) in the absence (1, 3) or presence (2, 4) of F-XIII.

produced. Therefore, the possibility was recognized that sialic acid affected the process of the cross-linked fibrin formation.

The turbidity of clots occurring from asialofibrinogen is higher than that of intact fibrinogen clots.[10] To investigate the relationship of such sialic acid-induced difference in this turbidity to the elasticity of clot gel, time-related changes in turbidity associated with clot formation and clot gel elasticity determined by thromboelastography were compared between fibrinogen and asialofibrinogen. As shown in Fig. 4A, the turbidity associated with reaction time was much higher for asialofibrinogen and sialic acid was found closely related to the apperance of turbidity reflecting the network structure of fibrin molecules. On the other hand, the results of thromboelastographic analysis disclosed little difference in the initiation time of reaction (*r*, about 2 min) between fibrinogen and asialofibrinogen, and no difference in the initiation time of gelation, the release stage of fibrinopeptides in the two was also confirmed by this results. However, the plateau of mean maximum amplitude (*ma*) reached for fibrinogen in the absence and presence of F-XIII, respectively, was 33.2 and 43.3, while these were 25.3 and 29.2 for asialofibrinogen. Briefly, obvious differences were noted in the thromboelastogram of fibrin gel formed from both types of fibrinogen. Furthermore, the maximum amplitude reached a plateau at about 45 min in fibrinogen clots. In asialofibrinogen clots, however, the maximum amplitude was scarcely time-related change in the absence of F-XIII, but tended to increase throughout the 2-h measuring time in the presence of F-XIII (Fig. 4B). These results may indicate that the amplitude of elasticity due to fibrin polymer gel and stabilized fibrin is dependent on the content of sialic acid and the action of F-XIII, and further, that higher elasticity generated in the action of F-XIII is affected by sialic acid in the fibrin molecule.

Next, the rigidity of clots was measured and compared using a hardness tester for stabilized-fibrin prepared by changing the concentration of both fibrinogens and the amount of thrombin. As shown in Table I, the value of asialofibrinogen-derived clots was lower than that of fibrinogen-derived clots used as controls regardless of conditions: rigidity of the former was found to be lower than that of the latter. Further, the absorbance of these stabilized fibrin clots at 350 nm was higher in asialofibrin (data not shown). This confirmed that clot turbidity did not necessarily reflect the gel rigidity.

As stated, the stabilized fibrin formation rate by F-XIII

Table I. Hardness Test of the Stabilized Fibrin Clots Derived from Fibrinogen and Asialofibrinogen

Thrombin (unit)	Fibrinogen		(kg)	Asialofibrinogen	
	0.5%	1%		0.5%	1%
10	0.272	0.508		0.121	0.218
20	0.336	0.679		0.213	0.309
40	0.419	0.760		0.276	0.354
80	0.495	0.851		0.350	0.397

The values represent the mean of three experiments. The stabilized fibrin derivatives were prepared from fibrinogen and asialofibrinogen with various concentrations of thrombin in the presence of purified F-XIII at 30 °C for 8 h. Each preparation was confirmed to be a complete cross-linked derivative as judged by SDS-PAGE using reducing agent. Details are in Materials and Methods.

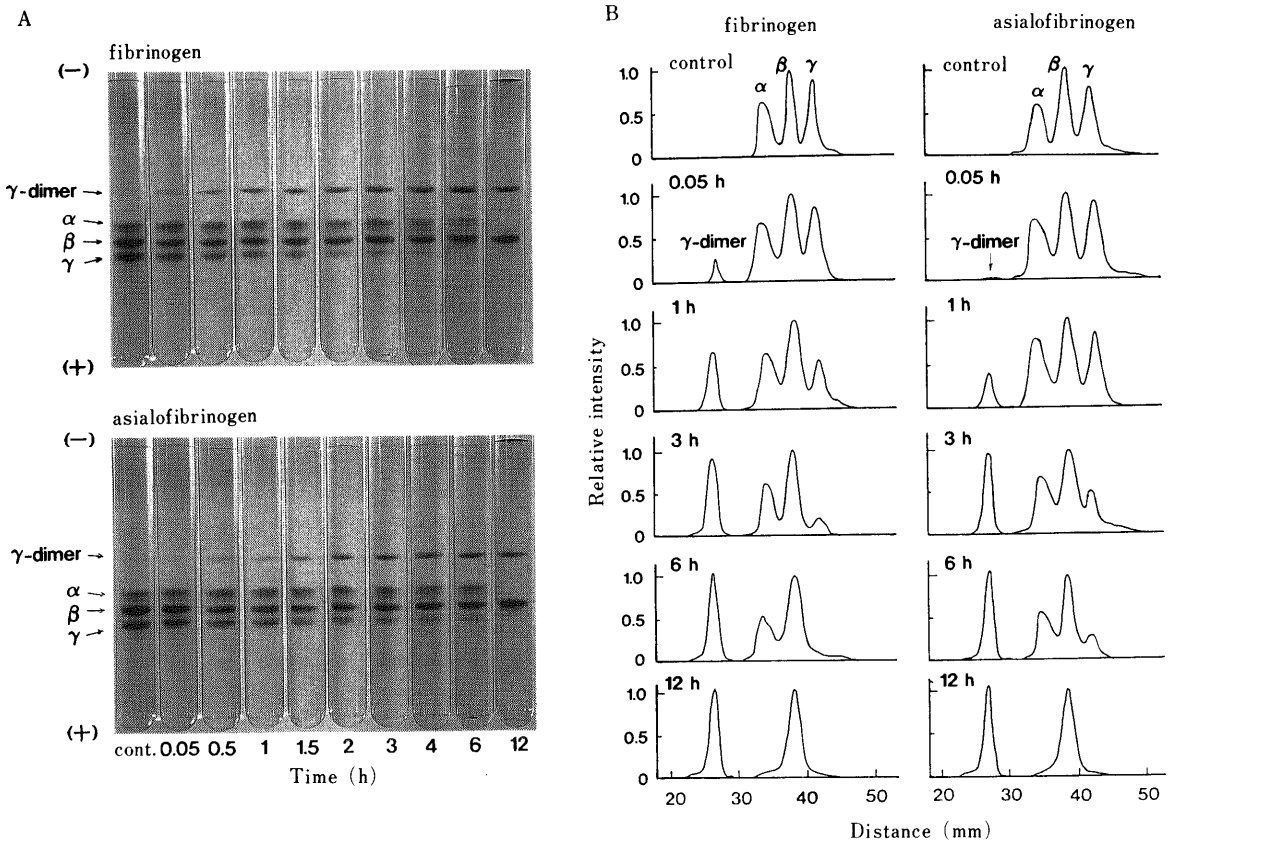

Fig. 5. γ-Dimer Formation Profiles of Fibrinogen and Asialofibrinogen on SDS-PAGE (A) and Densitometer (B)

Electrophoresis was carried out on about 20 μg of sample protein in each column (5% gel). The separated proteins were stained with Coomassie brilliant blue R-250. Densitometric analysis of separated proteins after electrophoresis was done at 20 mm per minute scanning rate and 8 mm slit width.

differed between fibrinogen and asialofibrinogen, so the cross-link formation rates of both fibrinogens in the presence of F-XIII were measured and compared by determining the production of γ-dimer using SDS-PAGE and a densitometer. As shown in Fig. 5A and B, after 3 min of reaction, γ-dimer began to appear in fibrinogen, but not in asialofibrinogen. It increased for the next 60 min in both materials, although production was higher in fibrinogen throughout the following 4 h. It was then almost completely replaced by dimer after 6 h of reaction in fibrinogen. However, γ-monomer was still detectable at this point of time in asialofibrinogen. These results indicate that sialic acid in fibrinogen influences the mechanism of fibrin intermolecular cross-link formation by F-XIII.

Calcium is considered unnecessary for thrombin-induced fibrinopeptide release from fibrinogen, but essential for polymerization of fibrin-monomer[26] and F-XIII-induced stabilized fibrin formation.[27] Fibrinogen has three high affinity calcium binding sites located in the D domains and amino-terminal disulfide knot (N-DSK), and binding calcium in these sites with high affinity is essential for the formation of fibrin clots,[28] that is, clot formation ability is greatly reduced by removal of this calcium with the addition of calcium chelating agent such as EDTA. Some low affinity calcium binding sites are also present in the molecule, one of them being sialic acid which influences fibrin assembly.[10] But calcium bound in low affinity sites is not significant in the process of fibrinogen-fibrin conversion when compared with high affinity binding calcium.

It has been demonstrated that fibrinogen, from which sialic acid has been removed and galactose exposed at the terminal end of the carbohydrate chain, is easily incorporated into reticuloendothelial tissues and degraded.[29] It may be a rational mechanism that fibrinogen reduced original functions, *i.e.*, stopping of bleeding from damaged tissue with rigid fibrin clots, by removal of sialic acid at terminal ends of carbohydrate regions as a result of catabolism during intravascular circulation is degraded and excreted.

Acknowledgement The authors would like to thank Professor H. Takahashi for his helpful comments on the manuscript and Dr. H. Iwai for his expert technical assistance.

References

1) R. F. Doolittle, *Annu. Rev. Biochem.*, **53**, 195 (1984); *idem*, *Adv. Protein Chem.*, **27**, 1 (1973).
2) K. Bailey, F. R. Bettelheim, L. Lorand and W. R. Middlebrook, *Nature* (London), **167**, 233 (1951).
3) M. Mihalyi, "Fibrinogen," ed. by K. Laki, Marcel Dekker, Inc., New York, 1968, pp. 61—86.
4) G. Koppel, *Thromb. Diath. Haemorrh.*, Suppl., **39**, 71 (1970).
5) L. Lorand and T. Gotoh, "Methods in Enzymology," Vol. XIX, ed. by G. E. Perlmann and L. Lorand, Academic Press, Inc., New York, 1970, p. 770; A. G. Loewy, K. Dunathan, R. Kriel and H. L. Woffinger, Jr., *J. Biol. Chem.*, **236**, 2625 (1961).
6) B. G. Langer, J. W. Weisel, P. A. Dinauer, C. Nagaswami and W. R. Bell, *J. Biol. Chem.*, **263**, 15056 (1988).
7) B. Bray and B. Alexander, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **28**, 321 (1969); K. Laki and N. Chandrasekhar, *Nature* (London), **197**, 1267 (1963).
8) P. A. Gentry and B. Alexander, *Arch. Biochem. Biophys.*, **173**, 50 (1976).
9) T. Diaz-Maurino, C. Castro and A. Albert, *Thromb. Res.*, **27**, 397 (1982).
10) C. V. Dang, C. K. Shin, W. R. Bell, C. Nagaswami and J. W. Weisel, *J. Biol. Chem.*, **264**, 15104 (1989).
11) C. Farios, M. Patorao de Macedo, A. C. Kempfer, J. P. Frontroth and M. Lazzari, *Thromb. Res.*, **51**, 325 (1988); J. L. Francis and D. J. Armstrong, *Haemostasis*, **11**, 215 (1982); J. Martinez, J. E. Palascak and D. Kwasniak, *J. Clin. Invest.*, **61**, 535 (1978); G. Green, J. M. Thompson, I. W. Dynack and L. Poller, *Br. J. Haematol.*, **34**, 427 (1976).
12) F. Streiff, P. Alexandre, C. Vigneron, J. Soria, C. Soria and L. Mester, *Thromb. Diath. Haemorrh.*, **26**, 565 (1971); M. S. Hansen and I. Schousboe, *Scand. J. Haematol.*, **33**, 9 (1984); L. Mester and L. Szabados, *Bull. Soc. Chim. Biol.*, **50**, 2561 (1968).
13) H. Maekawa, K. Yamazumi, S. Muramatsu, M. Kaneko, H. Hirata, N. Takahashi, N. B. de Bosch, Z. Carvajal, A. Ojeda, C. L. Arocha-Pinango and M. Matsuda, *J. Biol. Chem.*, **266**, 11575 (1991).
14) V. J. Simmonds and J. L. Francis, *Med. Lab. Sci.*, **44**, 119 (1987).
15) R. F. Doolittle, D. Schubert and S. A. Schwartz, *Biochim. Biophys. Acta*, **118**, 456 (1967).
16) J. McDonagh, W. G. Waggoner, E. G. Hamilton, B. Hindenach and R. P. McDonagh, *Biochim. Biophys. Acta*, **446**, 345 (1976).
17) L. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969); S. Iwanaga and S. Nakamura, *Rinsho–Kagaku*, **9**, 605 (1973).
18) M. Okude, Y. Morimoto and A. Akihama, *Jpn. J. Clin. Chem.*, **20**, 51 (1991).
19) W. Ruf, A. Bender, D. A. Lane, K. T. Preissner, E. Selmayr and G. Muller-Berghaus, *Biochim. Biophys. Acta*, **965**, 169 (1988).
20) H. Hartert, *Klin. Wochenschr.*, **326**, 577 (1948).
21) V. J. Marder and N. P. Schulman, *J. Biol. Chem.*, **244**, 2111 (1969).
22) B. Blombäck, B. Hessel, D. Hogg and L. Therkilden, *Nature* (London), **275**, 501 (1978).
23) W. Krakow, G. F. Endres, B. M. Siegel and H. A. Scheraga, *J. Mol. Biol.*, **71**, 95 (1972).
24) G. M. Fuller and R. F. Doolittle, *Biochem. Biophys. Res. Commun.*, **25**, 694 (1966).
25) M. L. Schwartz, S. V. Pizzo, R. L. Hill and P. A. McKee, *J. Biol. Chem.*, **246**, 5851 (1971).
26) A. Varadi and H. A. Scheraga, *Biochemistry*, **25**, 519 (1986); E. Mihalyi, *ibid.*, **27**, 967 (1988).
27) N. Nakamura, S. Iwanaga and T. Suzuki, *J. Biochem.* (Tokyo), **78**, 1247 (1975); K. R. Siebenlist and M. W. Mosesson, *Biochemistry*, **31**, 936 (1991).
28) C. V. Dang, R. F. Ebert and W. R. Bell, *J. Biol. Chem.*, **260**, 9713 (1985).
29) J. Martinez, J. Palascak and C. Peters, *J. Lab. Clin. Med.*, **89**, 367 (1977).