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In vitro fibrin clot formation and fibrinolysis using heterozygous plasma fibrinogen from γAsn319, Asp320 deletion dysfibrinogen, Otsu I

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Abstract

Introduction: We have reported a heterozygous dysfibrinogenemia, fibrinogen Otsu I, caused by the deletion of γAsn319 and γAsp320, which was originally identified in the dysfibrinogen Vlissingen/Frankfurt IV (V/FIV) associated with thrombosis. Unlike the V/FIV family, the Otsu propositus showed no thrombotic tendencies. To analyze the relationship between thrombosis and the heterozygous plasma variant fibrinogen, we used purified plasma fibrinogen from the Otsu patient and compared it with a normal control.

Materials and Methods: Thrombin-induced fibrin clot formation and clot structure were observed by fibrin polymerization and scanning electron microscopy, respectively. For *in vitro* observation of fibrinolysis, plasmin generation and clot lysis assays were performed by the addition of tissue type plasminogen activation (tPA) and plasminogen.

Results and Conclusions: Polymerization of Otsu was markedly impaired, while fibrin fibers were much thicker and the density of the bundles of fibrin fibers was less and porous compared with normal. Lysis of the Otsu clot was not significantly different from normal when a tPA and plasminogen mixture was overlaid onto the clots. For Otsu, the penetration of the tPA/plasminogen mixture into the clot was much faster than normal and the protection against plasmin cleavage was impaired, however, tPA-induced plasmin activation of the Otsu fibrin was slower than that of normal fibrin, resulting in a clot lysis of Otsu similar to normal.

Key words: dysfibrinogen, γ319, 320 deletion, fibrin clot formation, fibrinolysis, tissue-type plasminogen activator

Abbreviations: V/FIV, Vlissingen/Frankfurt IV; tPA,tissue-type plasminogen activator; FXIII,factor XIII; SEM,scanning electron microscopy; SDS-PAGE,sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Vmax,maximum rate; Lmax,maximum lysis

rate; HEPES:N- (2-hydroxy-ethyl) piperazine -N'- (2-ethanesulfonic acid);ECL,enhanced chemiluminescence

Introduction

Fibrinogen is a dimeric plasma glycoprotein, each half of the dimer being composed of three polypeptide chains, Aα, Bβ and γ. During blood coagulation, fibrinogen is converted by thrombin to an insoluble fibrin clot via a two-step process. Half-staggered, double-stranded protofibrils first form and then these protofibrils laterally aggregate to assemble into thick, multi-stranded fibers that branch to form a fibrin network [1]. We have reported a heterozygous dysfibrinogenemia, designated fibrinogen Otsu I caused by the deletion of γAsn319 and γ Asp320 [2], which was asymptomatic with neither bleeding nor thrombosis. The same heterozygous deletion was originally identified in the dysfibrinogen Vlissingen/Frankfurt IV (V/FIV) associated with thrombosis [3]. The V/FIV variant has only been reported in one large family of which there are at least 11 members afflicted with arterial or venous thrombosis (Thesis of Kelly A Hogan, University of North Carolina). Family members with this defect meet all criteria for thrombophilia associated with familial dysfibrinogenemia as determined by the Scientific and Standardization Committee on Fibrinogen of the International Society on Thrombosis and Hemostasis [4]. Hogan *et al*. synthesized a recombinant V/FIV fibrinogen and demonstrated that this deletion affected many functions associated with the C-terminal domain of the γ-chain [5]. In the recombinant V/FIV, calcium binding, fibrin polymerization, platelet aggregation and factor XIII-catalyzed cross-linking of fibrin were all disrupted [5]. The loss of these functions would typically be associated with bleeding, and not thrombosis. We speculate that the presence of heterodimers (molecules made of one normal and one variant γ-chain) might be necessary for the thrombotic phenotype, and purified plasma fibrinogen of the Otsu patient for *in vitro* studies of the relationship between delayed fibrinolysis and the γ-319, 320 deletion. To

examine impaired fibrinolysis in the context of the γAsn319 and γAsp320 deletion, we compared fibrin clot formation and clot lysis of the heterozygous Otsu fibrin (ogen) to that of a normal control.

Materials and Methods

Purification of plasma fibrinogen

Fibrinogen was purified from citrated plasma obtained from the propositus of Otsu and from a normal control (NC) subject (Japanese female, 28 years old) with informed consent. Purification was performed as described [6].

Thrombin-catalyzed fibrin polymerization

Thrombin-stimulated fibrin polymerization was performed in a final volume of 100 μl as described elsewhere [6]. Briefly, fibrinogen in 20 mM N- [2-hydroxy-ethyl] piperazine -N'- [2-ethanesulfonic acid] (HEPES) pH 7.4, 0.12 M NaCl (referred to as polymerization buffer) containing 1.0 mM $CaCl_2$ was mixed with 0.05 U/ml or 1 U/ml of human α-thrombin (Enzyme Research Laboratories, South Bend, IN) and changes in turbidity were monitored at an ambient temperature. The final concentrations of fibrinogens were NC; 0.45, Otsu; 0.45, and Otsu-1.34x; 0.60 mg/ml. The Otsu fibrinogen was also used at a 1.34-fold concentration of fibrinogen, 0.60 mg/ml, to obtain fibrin clots incorporating the same amounts of fibrinogen as normal, because the Otsu fibrinogen was only 74.4 % coagulable [7]. Each reaction was performed in triplicate and three parameters (lag period, maximum slope; Vmax and Δabsorbance over a 30 min period were obtained from the turbidity curves, as described elsewhere [6].

Scanning electron microscopy (SEM)

Samples for scanning electron microscopy (SEM) were prepared as described before [8]. Briefly, 10 μl of α-thrombin was added to 40 μl of fibrinogen solution from NC, Otsu and Otsu-1.34x and mixed by repeated pipetting. Polymerization proceeded in a moisture chamber at 37˚C for 3 h. The final concentrations of fibrinogen and the

α-thrombin were 0.40 (NC and Otsu) or 0.54 (Otsu-1.34x) mg/ml and 0.1 U/ml, respectively. The clots were fixed in 2.5 % glutaraldehyde overnight, stained with 1 % osmium tetroxide, critical-point dried, mounted, osmium plasma-coated at 5 nm thickness in an NL-OPC40 (Nippon Laser and Electronics Laboratory, Nagoya, Japan), and finally viewed on a JSM-6000F (Japan Electron Optics Laboratory Co. Ltd, Tokyo, Japan). Images were taken at 3000x or 20000x with a 15.0 kV accelerating voltage. Fiber diameters were measured using a vernier caliper on a 300 % enlargement from a photograph taken at a magnification of 20000x (n=20).

Factor XIIIa-catalyzed cross-linking of fibrin

Factor XIIIa-catalyzed cross-linking of fibrin was performed as described elsewhere [9]. A hundred nM of Factor XIII (FXIII, Enzyme Research Laboratories, South Bend, IN) was activated (FXIIIa) with the human α-thrombin (1 U/ml) for 60 min at 37˚C in the polymerization buffer containing 5 mM $CaCl_2$. The Otsu or NC fibrinogen (final concentration; 0.47 mg/ml) was reacted with FXIIIa (final concentration; 6.7 nM) and the human α-thrombin (final concentration; 0.07 U/ml) at 37˚C in the presence of 0.67mM calcium. The reactions were stopped at various time points (0, 1, 2, 3, 5, 20 min, 1, 2, 4 and 8 h) by adding an equal volume of sodium dodecyl sulfate (SDS) sample buffer containing 2-mercaptoethanol and boiling for 5 min. Samples equivalent to 4.7 μg of fibrinogen were separated by 8 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue R-250. To estimate the residual concentration of mutant- and wild-type γ-chains from Otsu patient fibrinogen after 8 h incubation, we performed densitometric analysis using Rapid Electrophoresis System (Helena Lab. Saitama, Japan).

Plasmin generation assay

The generation of plasmin from plasminogen by activation with a two-chain tissue type-plasminogen activator (tPA) was performed in 2 sets of experimental conditions as described by Wilhelm *et al*. [10] and Meh *et al*. [11]. In the first set of experiments, 100

μl of each purified fibrinogen (final concentration: NC; 0.45, Otsu-1.34x; 0.60 mg/ml) was polymerized with 1 U/ml of human α-thrombin in the polymerization buffer containing 1.0 mM $CaCl_2$ in the wells of a microtiter plate, and then incubated for 2 h at 37˚C. After the completion of polymerization, 100 μl of a mixture of tPA (Genentech, South San Francisco, CA), plasminogen (Roche Diagnostics GmbH, Mannheim, Germany), and chromogenic substrate; S-2251 (H-D-valine-leucine-lysine-p-nitroanilide, Chromogenix AB, Molngal, Sweden) in the polymerization buffer containing 1.0 mM $CaCl_2$, was overlaid onto each clot (final concentration: tPA; 1.2 nM, Plasminogen; 0.49 μM, S-2251; 0.44 mM), and the generation of plasmin was monitored as the increase in absorbance at 405 nm for 60 min. For the second set of experiments, 10 μl of human α-thrombin (final concentration; 1 U/ml) was added to a 190 μl mixture of the fibrinogens (NC; 0.23, Otsu-1.34x; 0.30 mg/ml), tPA (final concentration; 0.25 nM), plasminogen (final concentration; 0.21 μM), and S-2251 (final concentration; 0.44 mM) in the polymerization buffer containing 1.0 mM $CaCl_2$. The plasmin-specific changes in absorbance at 405 nm were obtained by the subtraction of the non-specific absorbance due to the turbidity of fibrin generation or the degradation of the S-2251. The reaction was performed in triplicate, and three parameters, lag period, the maximum rate (Vmax), and Δabsorbance over a 60 min period, were obtained from the absorbance curves.

Clot lysis assay

The clot lysis assay was performed with plasmin or a mixture of tPA and plasminogen in the absence or presence of FXIII using a modified procedure as described by Mullin *et al*. [12]. Briefly, in the absence of FXIII, 100 μl of purified fibrinogen was polymerized as described for the plasmin generation assay. In the presence of FXIII (final concentration; 2.0 nM), the FXIII was activated by the human α-thrombin (final concentration; 1 U/ml) for 60 min at 37˚C in the polymerization buffer containing 1 mM $CaCl_2$, and fibrinogen (final concentration; same as in the absence of the FXIII) was added and incubated at 37˚C for 2 h. After the completion of polymerization, 100 μl of

plasmin (Chromogenix AB, Molngal, Sweden) or a mixture of a tPA and plasminogen (tPA/plasminogen mixture) was overlaid onto each clot (final concentration: plasmin; 0.13 μM, tPA; 1.2 nM, plasminogen; 0.49 μM), and fibrinolysis was monitored as the decrease in turbidity at 350 nm for 120 or 240 min. Each reaction was performed in triplicate and three parameters, lag period, the maximum lysis rate (Lmax) and the 20 % lysis-time, which is the period until maximum absorbance (as 100 %) decreases to 80 %, were obtained from the turbidity curves.

SDS-PAGE and immunoblot analysis for lysis products.

SDS-PAGE analysis was performed to assess the difference of species of the degradation products and their amounts between the clots made from NC and Otsu-1.34x fibrinogen. Clots of the NC and Otsu-1.34x fibrinogen were made as described for the clot lysis assay with or without the addition of FXIII. After the completion of polymerization, 100 μl of a mixture of tPA and plasminogen (final concentration: tPA; 1.2 nM, plasminogen; 0.49 μM) was overlaid onto each clot. At various time points (0, 1, 2, 3, 4 and 7 h), lysis products were separated from the remaining clots by centrifugation at 15,000 rpm for 5 min, and added to an equal volume of non-reducing sample buffer. The samples (25μl) were separated on SDS-8 % polyacrylamide gels and stained with Coomassie Brilliant Blue R-250. To identify species of the degradation products, the sample for 7 h lysis, was separated on 8 % gel and its blots were developed with rabbit antihuman fibrinogen-D fragment or antihuman fibrinogen-E fragment antibody (both from Medical and Biological Laboratories, Nagoya, Japan) [13]. Cross-reacting species were visualized with horseradish peroxidase-conjugated goat antirabbit IgG antibody (MBL) and enhanced chemiluminescence (ECL) detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK). Blots were exposed on Hyperfilm-ECL (Amersham Pharmacia Biotech) [14].

Results

Thrombin-catalyzed fibrin polymerization

Thrombin-catalyzed fibrin polymerization (TCFP) was monitored as the change in turbidity at 350 nm. Representative turbidity curves are shown in Fig. 1. On the addition of 0.05 U/ml thrombin, we found that polymerization for the Otsu fibrinogen was markedly impaired as compared with NC, namely, a longer lag period (7.0 ± 0.5 min), a slower maximum rate (0.092 ± 0.203 Abs/min) and a smaller Δabsorbance (0.673 ± 0.042) than for NC (2.0 ± 0.5 min, 0.205±0.023 Abs/min and 1.108±0.132, respectively). However, for the Otsu-1.34x fibrinogen, the polymerization curve revealed a similar maximum rate (0.164 ± 0.052 Abs/min) and Δabsorbance (1.173 ± 0.078) to, but a longer lag period (5.0 ± 0.5 min) than NC (Fig. 1A). On the addition of 1 U/ml thrombin, all lag periods (NC; 0.5 ± 0.2, Otsu; 1.0 ± 0.3, Otsu-1.34x; 0.5 ± 0.2 min) were shorter than on the addition of 0.05 U/ml thrombin, and the curve for Otsu-1.34x (0.237 ± 0.033 Abs/min) showed a similar maximum rate to NC (0.283±0.029 Abs/min), but Δabsorbance for Otsu-1.34x (1.271 ± 0.082) over a 30 min period was greater than for NC (0.980 ± 0.027) (Fig. 1B).

Observation of fibrin clot and fibers by SEM

To clarify the difference in the ultrastructure of the fibrin clots among the NC, Otsu and Otsu-1.34x fibrinogens, we observed fibrin clots under SEM. The density of bundles of fibrin fiber in the Otsu and Otsu-1.34x clots were less and the fibers' diameters were larger (the Otsu; 176 ± 23, Otsu-1.34x; 184 ± 29 nm) than in NC clot (97 ± 16 nm) (n=20). A few truncated fibers were observed in the Otsu and Otsu-1.34x clots (Fig.2).

Factor XIIIa-catalyzed cross-linking of fibrin

FXIII-catalyzed cross-linking of fibrin α- and γ-chains was performed with FXIIIa and thrombin, and the reaction products were observed using SDS-PAGE analysis as described in "Materials and Methods". For the initial phase of the cross-linking process,

both with the NC and Otsu fibrins, γ-γ dimer and α-polymer bands were evident after 2 and 5 min, respectively, and both bands increased in a time dependent manner (Fig. 3 A, C). In order to assess the influence of fibrinopeptide B (FPB) release to FXIIIa-catalyzed cross-linking, we observed the cross-linking process for 1 to 8 h. For not only the NC but also Otsu fibrinogen, FPB release was completed by 4 h. While a slight amount of the γ-chain from NC was still observed at 8 h, the α-chain was completely cross-linked into α-polymer after 4 h incubation. However, a significant amount of γ-chain of the Otsu fibrinogen remained at 8 h, and the α-chain also clearly remained. Densitometric analysis of Otsu fibrinogen before cross-linking showed that the mutant γ-chain comprised only 0.51 of the wild type. However, after 8 h incubation, the amount of non cross-linking mutant γ-chain was 3.2-fold that of normal γ-chain (Fig. 3 B, D).

Plasmin generation assay

The plasmin generation assay was performed as described in "Materials and Methods". Plasmin activation was determined with the rise in absorbance at 405 nm. Representative absorbance curves for 2 sets of experimental conditions are shown in Figs. 4A and B. We measured three parameters, the lag period, the maximum rate (Vmax), and the Δabsorbance over a 60 min period. For the first set of plasmin generation mixtures of tPA and plasminogen overlaid on the fibrin clot, the lag period of Otsu-1.34x (<0.5 min) was shorter and the Vmax (0.022 ± 0.002 Abs/min) was smaller than that for NC (15 ± 2 min, 0.028 ± 0.001 Abs/min), but the Δabsorbance of Otsu-1.34x (0.822 ± 0.022) was not significantly different from NC (0.842 ± 0.030)(Fig. 4A). For the second set of plasmin generation, thrombin, fibrinogen, tPA and plasminogen were mixed simultaneously and showed that the Vmax of Otsu-1.34x (0.011 ± 0.003 Abs/min) was decreased and Δabsorbance (0.365 ± 0.033) was lower than those of NC (0.015 ± 0.002 Abs/min, 0.621 ± 0.032) (Fig. 4B).

Clot lysis assay

The clot lysis assay was performed as described in “Materials and Methods”. The clot lysis process was monitored with the decrease in absorbance at 350 nm. Representative lysis curves for 4 sets of experimental conditions are shown in Figs. 5A-D. From the curve, we measured the lag period, the maximum slope (Lmax) and the 20% lysis time (Table 1). In the absence of FXIII, for lysis initiated with the addition of plasmin, the turbidity of Otsu-1.34x immediately decreased and the Lmax of Otsu-1.34x was 4-fold faster than that of NC ($p < 0.01$) (Fig. 5A). Clot lysis initiated with the tPA/plasminogen mixture showed that the lag period and the 20% lysis-time of Otsu-1.34x was slightly shorter than for NC, and the Lmax of Otsu-1.34x was slightly faster than that of NC (Fig. 5B). In the presence of FXIII (Figs. 5C and D), cross-linked fibrins showed a similar lysis curve to that in the absence of FXIII except for having longer lag periods.

SDS-PAGE and immunoblot analysis for the lysis products

SDS-PAGE analysis was performed to assess the difference of species of the degradation products and their amounts between the clots made from NC and Otsu-1.34x fibrinogen. The degradation products were separated from the remaining clots by centrifugation. As depicted in Fig 6, in the absence of FXIII (Figs. 6A and B), some of the fibrinogen not incorporated into the clot remained Otsu-1.34x at 0 time. We considered that these portions of Otsu fibrinogen corresponded to fibrinogen composed of two variant γ-chains, as previously reported [5]. Plasmic fragments of X, $D_{1, 3}$ and E were observed in the NC and Otsu-1.34x clots, and additional D_2 fragments were shown only in the Otsu-1.34x clot. The degradation products of the Otsu clot were similar to the NC clot, although the amount of X in Otsu was slightly increased as compared with NC. In the presence of FXIII (Figs. 6C and D), some of the fibrinogen not incorporated into the clot also remained in Otsu-1.34x at 0 time. Plasmic fragments of D dimer, $D_{1, 3}$ and E were observed in the NC and Otsu-1.34x clots, and additional D_2 fragment were

shown in the Otsu-1.34x clot. The degradation products of the Otsu clot were similar to NC with cross-linking fibrin. Lower molecular bands under D_3 in each lane (*) did not react with antihuman fibrinogen-D antibody or fibrinogen-E antibody (data not shown). Species of the degradation products and their amounts were not significantly different between NC and Otsu-1.34x except for the D_2 band, which was supposedly due to variant fibrinogen.

Discussion

We studied fibrin clot formation and fibrinolysis using a heterozygous variant fibrinogen, Otsu I, caused by a γAsn319 and γAsp320 deletion [2]. Studies with the same variant fibrinogen V/FIV, in which the fibrinogen level was in the normal range (3.1 g/L), demonstrated impaired polymerization, reduced calcium binding and reduced ADP-induced platelet aggregation [3, 15], and with the recombinant fibrinogen analogous to fibrinogen Otsu and V/FIV, which comprised only variant molecules, demonstrated complete loss of these functions along with FXIIIa-catalyzed cross-linking [5]. The Otsu fibrinogen had a level of plasma fibrinogen just below that of the normal range (1.8 g/L) measured by an immunologic method [7]. Polymerization of purified plasma fibrinogen Otsu, a mixture of wild-type and variant molecules, was impaired, but FXIIIa-catalyzed formation of γ-γ dimers in the initial phase was similar to that of the NC. After 8 h cross-linking, the component of non cross-linking γ-chain of the Otsu type indicated that the amount of mutant γ-chain was 3.2-fold that of wild type. This finding suggests the variant molecule of the γ-chain can not cross-link into normal γ-chain. We speculate that the loss of γ319 and γ320 residues changes the tertiary structure of the C terminal portion of the mutant γ-chain, resulting in impairment of the cross-linking sites, γ398Glu (acceptor) and γ406Lys (donor). Furthermore, significant amount of the non cross-linked α-chain of the Otsu fibrinogen also remained, suggesting that formation of an α-γ dimer which might be considered an intermediate of α-polymer was impaired in

comparison with such formation in the NC.

Moreover, using a 1:1 mixture of normal and variant fibrinogens as an *in vitro* model for plasma fibrinogen from a heterozygous individual, polymerization of the plasma protein was markedly impaired relative to the mixture [5]. Based on these results and findings we speculate that the presence of heterodimers might be significant in clinical cases where the presence of the abnormal molecules might lead to thrombosis in several members of the V/FIV family. Namely, both normal and variant fibrinogens are present in the plasma, incorporated into the clot, and result in an alteration of clot formation and/or dissolution by plasmin, finally leading to symptoms of thrombosis.

To analyze the relationship between delayed fibrinolysis and the γ319, 320 deletion, we purified heterozygous plasma fibrinogen from the Otsu patient and performed clot formation and clot lysis assays. From observation of the fibrin clot structures under SEM, the fiber diameter of Otsu-1.34x was not different to that of Otsu, and much thicker than that of NC, whereas the density of the bundles of fibrin fibers of Otsu and Otsu-1.34x were less and more porous than that of NC.

It has been reported that the complete lack of plasminogen is not association with thrombosis but with ligneous conjunctivitis [16]; however, it is also well known that the plasminogen-plasmin system is principally involved in fibrinolysis both *in vivo* and *in vitro*. The clot lysis assay was performed with a plasmin or a tPA/plasminogen mixture overlaid onto each clot. The clot lysis rate with a plasmin of the Otsu-1.34x clot was much faster than that of NC in the absence or presence of FXIII. Clot structure observated by SEM suggested that penetration of the overlaid solution into the Otsu clot may be much faster than that into NC. In addition, it is known that fibrinolysis with plasmin is protected from cleavage in the presence of calcium ions, however deleted residues, γ319 and γ320, were located within one of the known high affinity calcium binding sites of fibrinogen [17]. Therefore the Otsu clot had reduced protection from plasmin cleavage and was lysed readily by plasmin. Similar data were obtained from

plasma fibrinogen of V/FIV [3] and recombinant γ 319, 320 deleted fibrinogen [5]. On the clot lysis with the tPA/plasminogen mixture added onto the clots, the lag period, 20 % lysis-time or maximum lysis rate of Otsu in the absence of FXIII was no significant differences between Otsu and NC. We considered that for the Otsu clot, the penetration of tPA/plasminogen was much faster and the calcium protection against plasmin cleavage was also more reduced than for NC, whereas the tPA binding to the Otsu fibrin was impaired, because the sequence γ312-324 was identified as one of the three tPA-binding sites on the fibrin molecule [18, 19], especially, γ-Asp320 is reported to be an important residue for the binding of tPA to fibrin [10]. Therefore, tPA-induced plasmin generation of the Otsu fibrin was slower than that of NC, resulting in a clot lysis rate of Otsu similar to that of NC. Reduced tPA-induced plasmin generation was directly confirmed by the plasmin generation assay, where the tPA/plasminogen was simultaneously mixed with fibrinogen and thrombin. In the presence of FXIII, the lag period of clot lysis was longer than that in the absence of FXIII, however, maximum lysis rate and 20 % lysis-time were similar to those in the absence of FXIII. These results suggested that the tPA/plasminogen solution seeps into cross-linked fibrin clots slower than into non-cross-linked fibrin clots. Supporting the results of the SDS-PAGE analysis, the degradation products of the clot lysis were not significantly different between NC and Otsu fibrin. Finally, although our *in vitro* clot lysis condition was greatly restricted, for the clot from Otsu plasma fibrinogen, fibrinolysis induced by tPA/plasminogen was not significantly delayed, leading to the conclusion of no association between dysfibrinogenemia and thrombosis.

To investigate whether the γ319, 320 deletion could lead to thrombosis, Hogan *et al* used gene targeting to generate mice with a comparable V/FIV human mutation [20]. Brother-sister mating produced mice heterozygous and homozygous for the deletion on a mixed 129 used for phenotypic studies. Heterozygous mice were not different from normal, although all homozygous mice suffered from neonatal bleeding and 47 % died

within the first 48 h of life. In studies of the pathology, 2- to 3-month old mice of each genotype showed no signs of fibrin deposition or hemorrhage from spleen, liver and lungs [20]. Furthermore they found that the heterozygous mice had approximately half and the homozygous mice one-eight the fibrinogen levels of wild-type mice, suggesting that the level of the variant γ-chain in plasma was substantially lower than that of the normal γ-chain, and associated that both mice of homozygous and heterozygous genotype revealed no thrombosis [20]. These results with mice confirmed our *in vitro* data and did not support that heterozygous patient with γ319, 320 deletion might be associated with thrombosis.

Approximately 20 % of the reported dysfibrinogens have been associated with thrombosis [2], but to demonstrate that a mutation found in fibrinogen directly causes the phenotype has been difficult. To establish a cause-effect relationship, the same mutation must be found in multiple families, the presence of the mutation must strongly correlate with thrombosis, and ideally, *in vitro* analysis should provide a biochemical explanation for the phenotype. To date, the best evidence linking dysfibrinogens with thrombophilia is the families with the AαArg554→Cys mutation (AαR554C). Five independent families with AαR554C-albumin binding have been identified and, strikingly, all probands have presented with thrombosis [2]. Additionally, *in vitro* studies with the AαR554C fibrinogen demonstrate that impaired fibrinolysis, abnormal rigidity of the clots, and an increased cross-linking potential likely explain the thrombosis and embolism seen in the families afflicted [21-26]. Some family members with the homozygous BβA68T mutation [27], presented with striking thromboembolic disease. The BβA68T variant fibrin was found to have a significant reduction in the binding affinity for thrombin compared to normal fibrinogen. Since low-affinity nonsubstrate thrombin binding sites are in the fibrin E domain, it is assumed that a complete absence of low-affinity thrombin binding sites in the BβA68T homozygotes, contributes to reduced thrombin affinity, and then decrease mitigating thrombin procoagulant effects *in*

vivo remain to be fully explored, accordingly a markedly thrombophilia [28]. In BβR14C- and BβR44C-albumin binding families [29], the defective binding of tPA to the fibrin moiety of the abnormal fibrinogen may cause abnormal tPA-mediated plasminogen activation and prolongation of blood clot lysis. An impairment of fibrinolysis in these cases was associated with thrombosis [30].

Further, several dysfibrinogenemia with thrombosis have been reported [2, 31], however, thrombosis is often found in only one family with few members or in some of the dysfibrinogenemia with same variants or same mutation sites. The exact mechanisms for thrombosis in these cases are unclear, suggesting that confounding factors might contribute to the thrombotic condition in these dysfibrinogenemia. Actually, no association between thrombophilia and protein C, protein S, or Anti-thrombin III had been studied in the V/FIV families, but other thrombotic factors like Factor V Leiden not, suggesting that the presence of confounding factors contributing to the thrombotic phenotype were not excluded. Besides studies of the Otsu family members are not available for additional studies for without consensus, it is difficult to draw many conclusions and comparisons *in vivo*. However, our *in vitro* studies could not confirm a relationship between impaired fibrinolysis and the γ319, 320 deletion. In particular, clot lysis of the Otsu fibrinogen is not impaired compared to that of NC.

In conclusion, we found dysfibrinogen Otsu I caused by the two-residue deletion γAsn319 and γAsp320, which was originally identified in the dysfibrinogen Vlissingen/Frankfurt IV associated with thrombosis. To analyze the relationship between delayed fibrinolysis and the γ319, 320 deletion, we performed clot formation and clot lysis analyses for purified plasma fibrinogen from the Otsu patient and normal control. Our *in vitro* data indicated that the variant fibrinogen of γ319, 320 deletion was not impaired in fibrinolysis compared to the normal, suggesting that the variant is not associated directly with thrombosis, and these conclusions are coincident with data

obtained from heterozygous mice.

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Table legend

Table 1. Three parameters of clot lysis assay. Three parameters, lag period, the maximum lysis slope (Lmax), and the 20 % lysis time, were obtained from the turbidity curves and the data represent the mean ± standard deviation of triplicate experiments.

Figure legends

Figure 1. Thrombin-catalyzed fibrin polymerization. Thrombin-catalyzed fibrin polymerizations were performed at 0.45 mg/ml of fibrinogen with 0.05 U/ml of α-thrombin (A) or 1 U/ml (B). The change in turbidity with time was monitored at 350 nm and representative polymerization curves from triplicate experiments are shown for NC (●) , Otsu (▲) and Otsu-1.34x (◆)

Figure 2. Scanning electron microscopy of fibrin clots formed using thrombin. The NC (A), Otsu (B) and Otsu-1.34x (C) fibrinogens were polymerized using 0.1 U/ml of α-thrombin. All clots were fixed, stained, critical-point dried and mounted (as specified in Materials and Methods). The bar represents 10 μm.

Figure 3. Factor XIIIa-catalyzed cross-linking of fibrin. Time-dependent FXIIIa-catalyzed crosslinking of fibrin was separated using 8 % SDS-PAGE under reducing conditions and stained with Coomassie Brilliant Blue R-250. The initial phase of cross-linking process (0 to 20 min-incubation) are A (NC) and C (Otsu) and the latest phases of 1 to 8 h-incubation are B (NC) and D (Otsu). The individual fibrinogen chains (α, β, γ, cross-linked γ-γ dimer and cross-linked α-chain polymers) are indicated on the right side of the gels. The molecular markers (250, 150, 100, 75 and 50 kDa; from top to bottom) were run in lane M.

Figure 4. Plasmin generation from plasminogen with tPA. Plasmin generation assays were performed in 2 sets of experimental conditions and the increase in absorbance at 405 nm was observed. Purified fibrinogens were polymerized with thrombin. After the completion of polymerization, plasmin generation was initiated with a mixture of tPA, plasminogen and S-2251 overlaid onto the clots (A). Plasmin generation is shown for the co-addition of the fibrinogens, thrombin, plasminogen, t-PA and S-2251 (B). Representative absorbance curves from triplicate experiments are shown for NC(●), and Otsu-1.34x (▲).

Figure 5. Clot lysis with plasmin. Clot lysis with plasmin was performed in 4 sets of experimental conditions and a decrease in the turbidity at 350 nm was observed. Fibrinogens of NC and Otsu-1.34x were polymerized with 1 U/ml of human α-thrombin and after the completion of polymerization, either plasmin (A, C) or a mixture of tPA and plasminogen (B, D) was overlaid onto clots. Clot lysis was performed in the absence (A, B) or presence (C, D) of FXIII. Representative curves from triplicate experiments are shown for NC(●) and Otsu-1.34x (▲).

Figure 6. SDS-PAGE analysis for the clot lysis products with tPA-induced plasminogen activation. The lysis products were separated from the remaining clots by centrifugation and resolved by SDS-PAGE under non-reducing conditions and stained with Coomassie Brilliant Blue R-250. In the absence of FXIII; A (NC) and B (Otsu-1.34x), presence; C (NC) and D (Otsu-1.34x).
X; 200 kDa, reacted with anti fibrinogen-D and -E antibody, $D_{1\text{-}3}$; 100-70 kDa, reacted with anti fibrinogen-D antibody, E; 50 kDa, reacted with anti fibrinogen-E antibody, DD; 200 kDa, reacted with anti fibrinogen-D antibody, *; reacted with neither anti fibrinogen-D nor E antibody.

Table 1. Three parameters of clot lysis assay

		Plasmin onto clots			tPA/Plasminogen onto clots		
		lag period (min)	Lmax (Abs/min)	20% lysis time (min)	lag period (min)	Lmax (Abs/min)	20% lysis time (min)
XIII (-)	NC	30 ± 5	0.009 ± 0.002	32 ± 2	53 ± 5	0.011 ± 0.003	42 ± 3
	Otsu-1.34x	0 ***	0.038 ± 0.005 **	6 ± 2 ***	50 ± 5	0.015 ± 0.002	33 ± 5
XIII (+)	NC	30 ± 3	0.008 ± 0.002	30 ± 4	130 ± 12	0.011 ± 0.003	45 ± 4
	Otsu-1.34x	5 ± 1 ***	0.032 ± 0.004 **	14 ± 3 *	90 ± 6 *	0.013 ± 0.004	37 ± 3

Unpaired Student *t*-test for Otsu-1.34x versus NC (*p<0.05, **p<0.01, ***p<0.001)

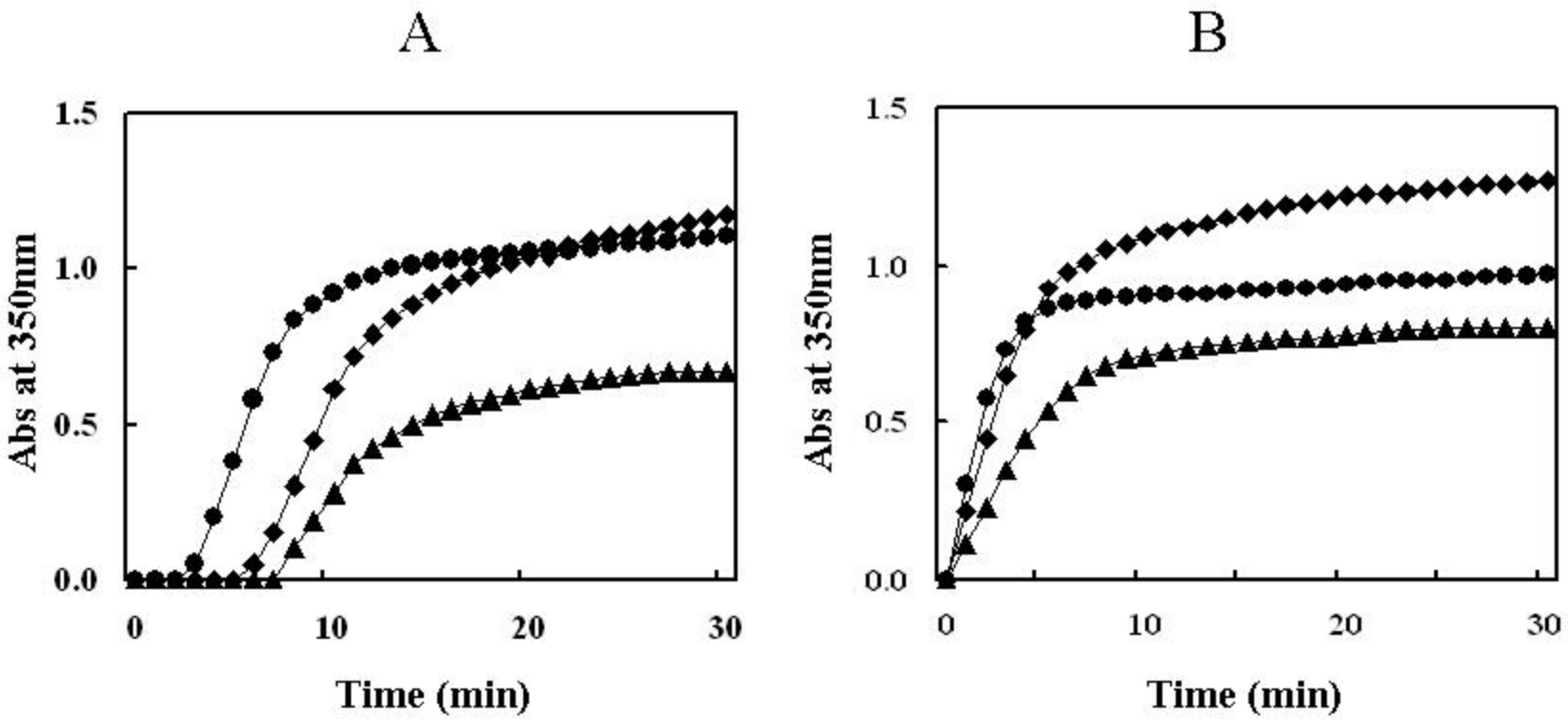

Fig. 1

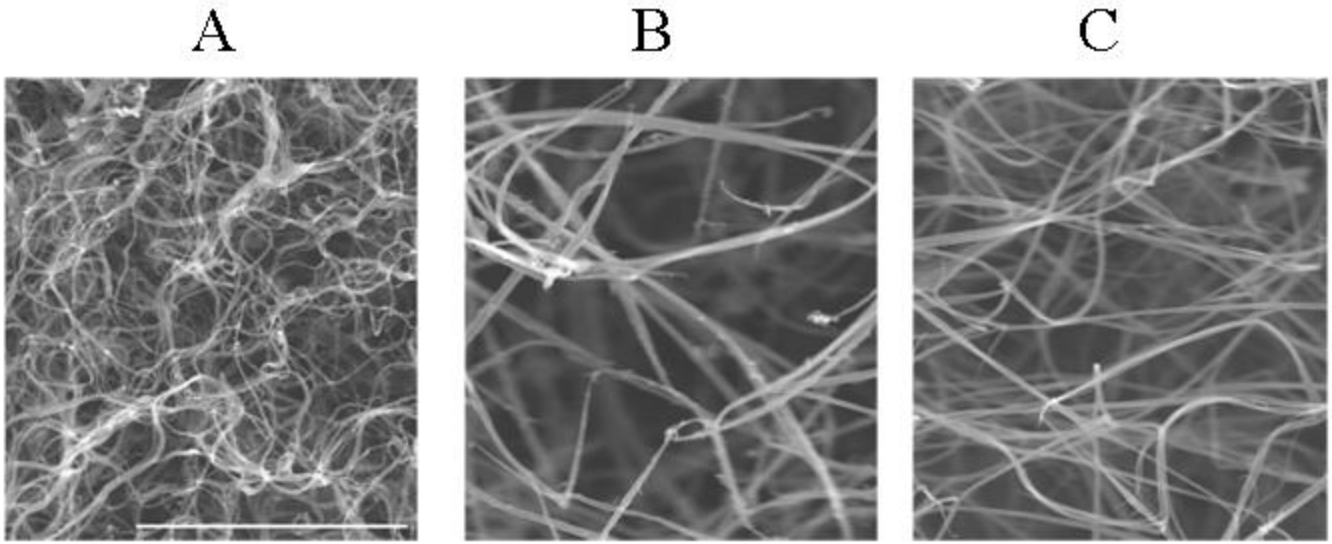

Fig. 2

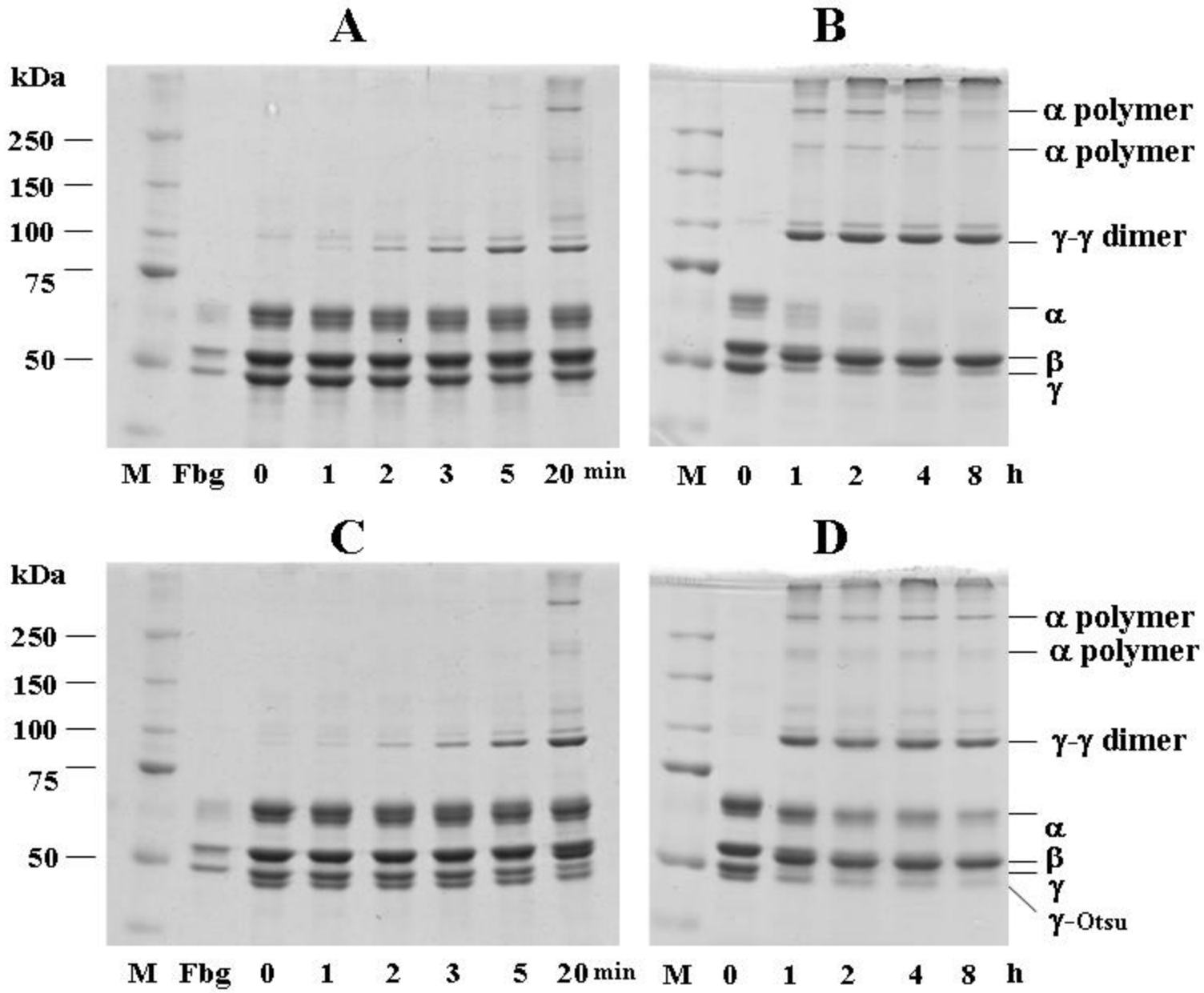
A
B
C
D
kDa
250
150
100
75
50
M Fbg 0 1 2 3 5 20 min
M 0 1 2 4 8 h
α polymer
α polymer
γ-γ dimer
α
β
γ
γ-Otsu

Fig. 3

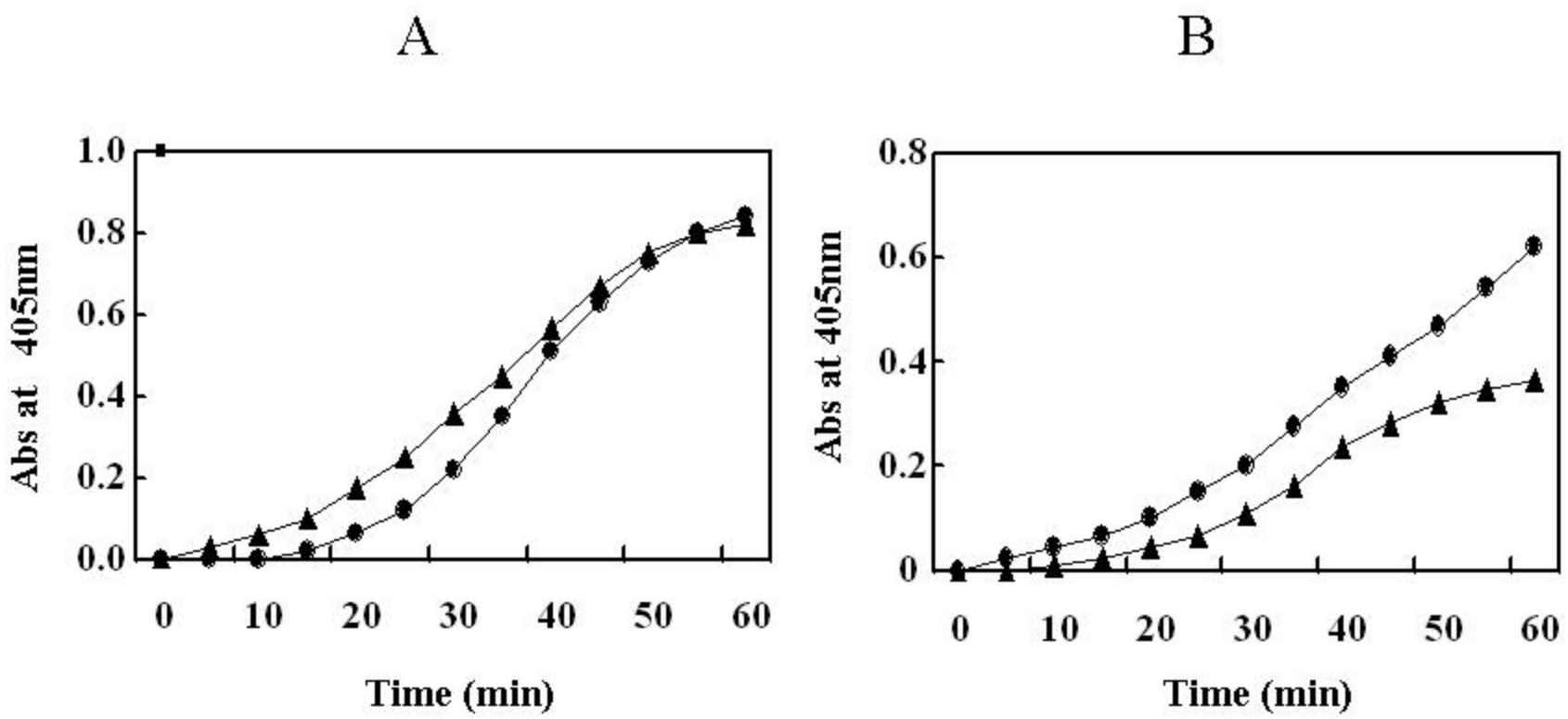

Fig. 4

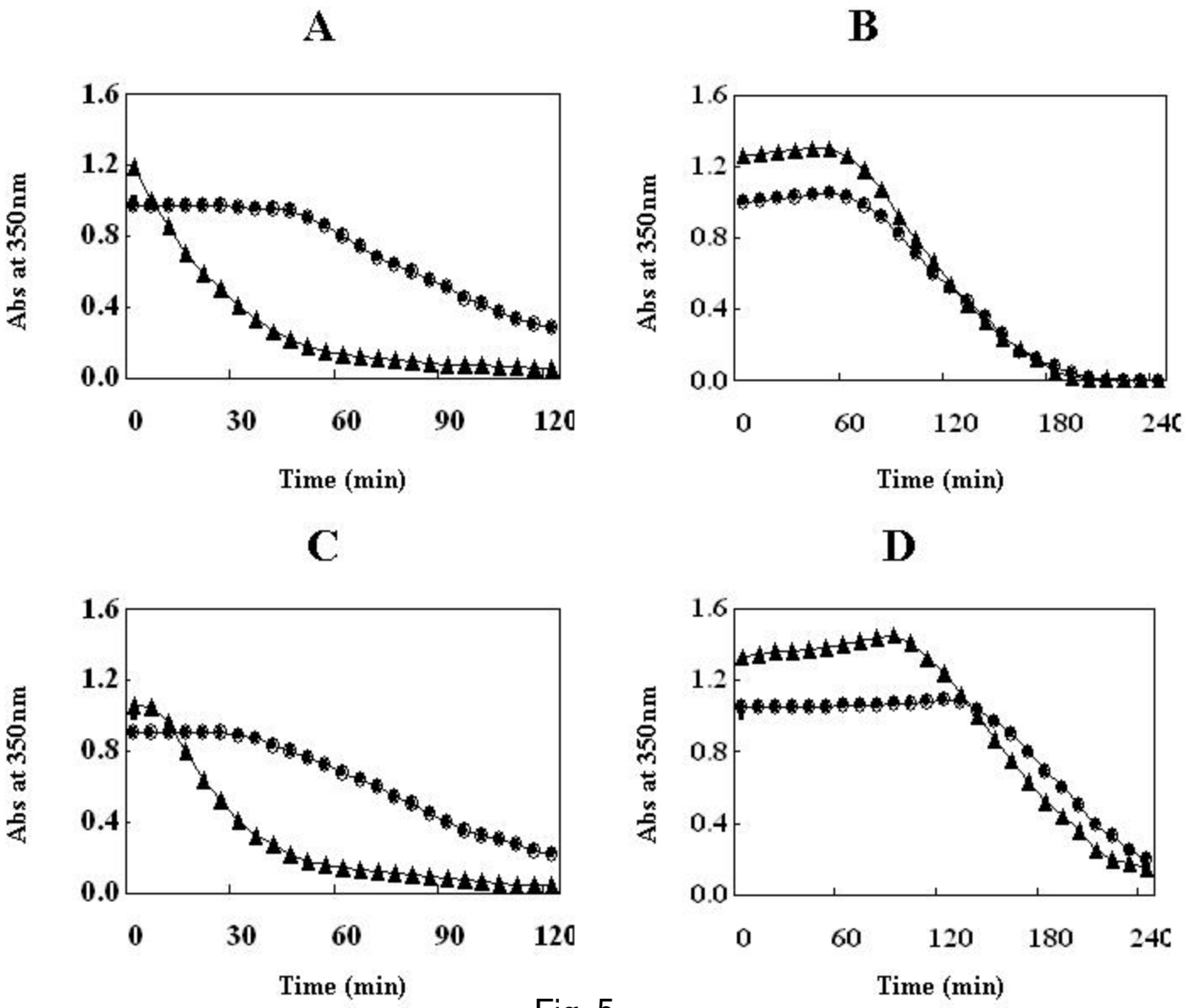

Fig. 5

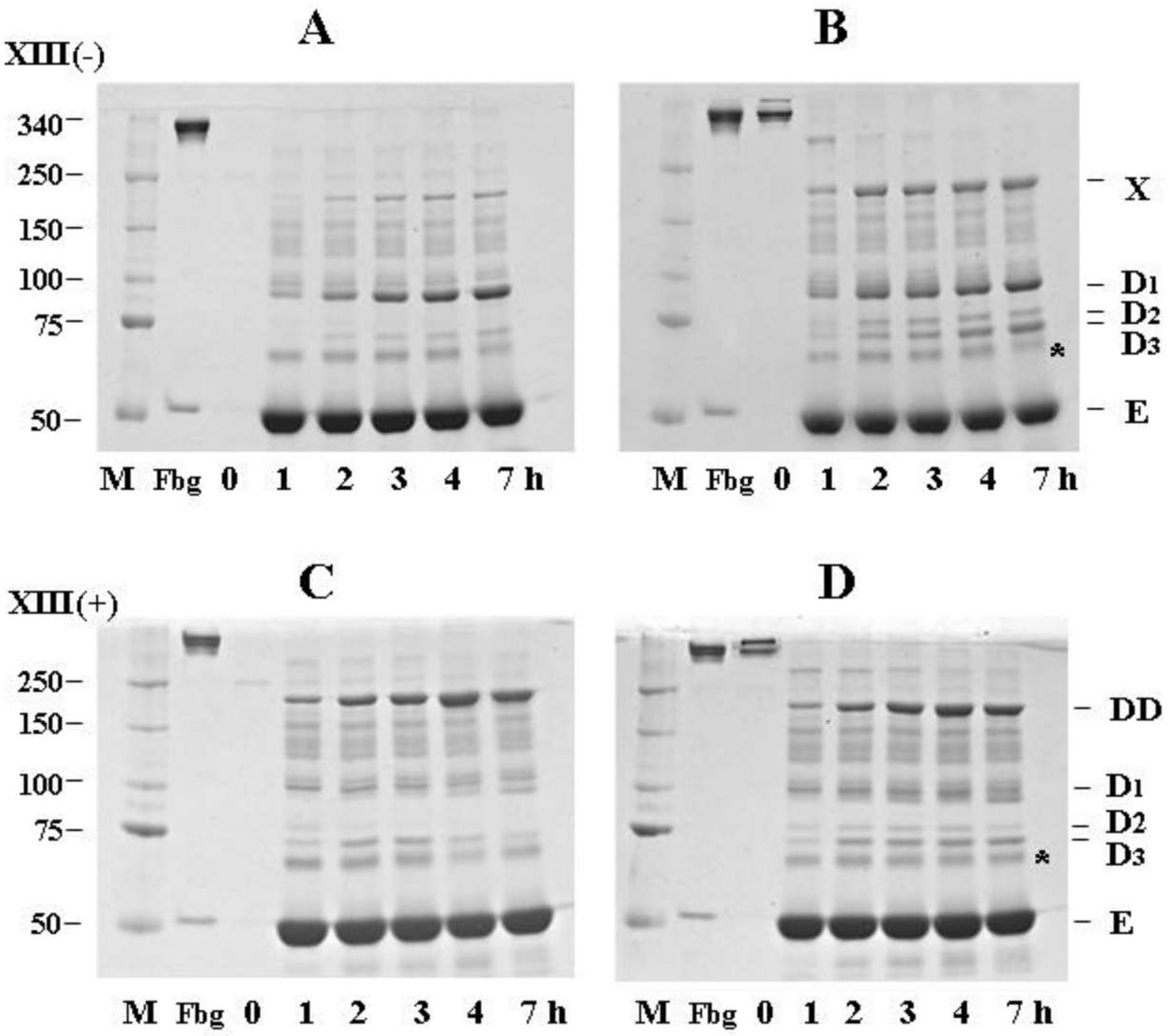

Fig. 6