

FIBRIN (OGEN)/FIBRINOGEN DEGRADATION PRODUCTS—INTERACTION WITH POLYMER SUBSTRATE AND PLATELETS

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ABSTRACT

The effect of three proteases viz plasmin, trypsin and thrombin on fibrinogen molecule and the interaction of the resultant products with an artificial surface and subsequently with washed platelets using labelled fg and SDS-PAG electrophoresis are reported. The modulation of platelet-surface interaction by these proteolytic products of fibrinogen is also reported.

INTRODUCTION

PROTEIN adsorption at interfaces in contact with blood plays an important role both in hemostasis and in surface-initiated thrombogenesis. Vroman *et al.*^{1,2} concluded in general that blood and plasma deposit proteins within seconds of contact with an artificial surface; often, fibrinogen (fg) is deposited early and predominantly. Physiologically, the fate of fibrinogen at interfaces is most significant because the platelets, at least *in vitro*, adhere preferentially where surfaces have adsorbed fibrinogen^{3,4}.

The fg molecule is composed of three pairs of non-identical peptide chains, A α , B β and γ , and its overall structure^{5,6} is therefore (A α , B β , γ)₂. Proteolytic enzymes like plasmin⁷, trypsin⁸ and thrombin⁹ have been powerful tools in the search of fibrinogen structure. They hydrolyze the fg at specific cleaving sites, which are needed for activating or lysing the molecule. In this paper, we studied the effect of three proteases, plasmin, trypsin and thrombin on fg molecule and the interaction of the resultant products (FDP or fibrinogen) with an artificial surface and subsequently with washed platelets using labelled fg and SDS-PAG electrophoresis. Further we report on the modulation of platelet-surface interaction by these proteolytic products of fg.

MATERIALS AND METHODS

Polycarbonate was used as the substrate in these studies. Proteases used were thrombin (2000 NIH units per mg protein, Sigma) and plasmin (4.1 units/mg protein, Sigma). Fibrinogen (human fraction I, 95% protein clottable, Sigma Co.), and other chemicals used were of the analar grade. Radiolabelled fibrinogen was

obtained from Amersham, England as iodinated (¹²⁵I) human fibrinogen.

Adsorption procedures

Polycarbonate films (2 × 3 cm) were prepared as reported earlier¹⁰. The films were then exposed to 220 mg% fibrinogen solution in 0.1 M phosphate buffer, (pH 7.4) containing 7% (0.51 μ curie/ml) ¹²⁵I labelled fibrinogen ensuring air/water interface¹⁰. Similarly other sets of films were prepared by exposing them to fg solution containing washed platelets (1 × 10⁶ platelets/ml) and proteases (25 mg% trypsin or plasmin or 0.5 units/ml thrombin), which were added separately inside the media to reduce the air/H₂O interface. Experiments were run for about 24 hr, to achieve an equilibrium. The films were taken out shaken and rinsed thoroughly with phosphate buffer. The films were counted employing a γ -counter and the surface concentration of fg λ (μ g cm⁻²) were calculated^{11,12}. The desorption kinetics of 3 hr pre-adsorbed surfaces were also carried out in fg solution and were quantitated.

Platelet adhesion studies with washed platelets

Calf blood platelets were isolated within 2 hr after collection, from citrated blood¹³ and suspended in tyrode solution¹⁴ for the adhesion studies. Platelet adhesions to PC surfaces were measured using platelets suspension (approx. 1.0 × 10⁶ platelets per ml) containing 220 mg% fg and varying concentrations of trypsin or thrombin. Platelet suspensions demonstrated above were exposed to the polymer surface for 15 minutes at room temperature (~ 30°C), rinsed with 0.1 M phosphate buffer, (pH 7.4), fixed the platelets with 2.5% glutaraldehyde, stained with Coomassie Blue G and were counted using an optical microscope.

Sodium dodecyl sulphate gel electrophoresis (SDS-PAGE)

PAGE was used to separate, identify and quantitate the adsorbed proteins^{15,16} from a mixture of proteins, after desorbing them using Triton X-100.

The dried PC films were exposed to 220 mg% fibrinogen solution in 0.1 M phosphate buffer, pH 7.4, avoiding the air-water interface¹⁰. After 3 hr, each film was taken out and rinsed with buffer thoroughly to remove all unadsorbed fibrinogen. The experiments were similarly repeated with trypsin (25 mg%) and thrombin (0.5 units/ml), infused fg-polymer system.

The sample preparation for PAG electrophoresis has been indicated earlier¹⁷. In brief, for desorbing, the proteinated films were placed in a beaker containing 10ml of 0.1 M Tris-HCl buffer (pH 8.5) with 1% Triton X-100. The films were incubated at room temperature ($\sim 30^{\circ}\text{C}$) for 16 hr with repeated shaking. The solution was then concentrated, and SDS-PAGE of desorbed proteins and proteins in media was performed on 7% gels containing 1% SDS using the method of Laemmli¹⁸.

RESULTS AND DISCUSSION

Figure 1 indicates that plasmin and trypsin has dramatically reduced the surface concentration of the hydrolytic products, but with platelets and these proteases, an initial enhanced fg/FDP surface binding is observed as shown in figure 2. A part of the

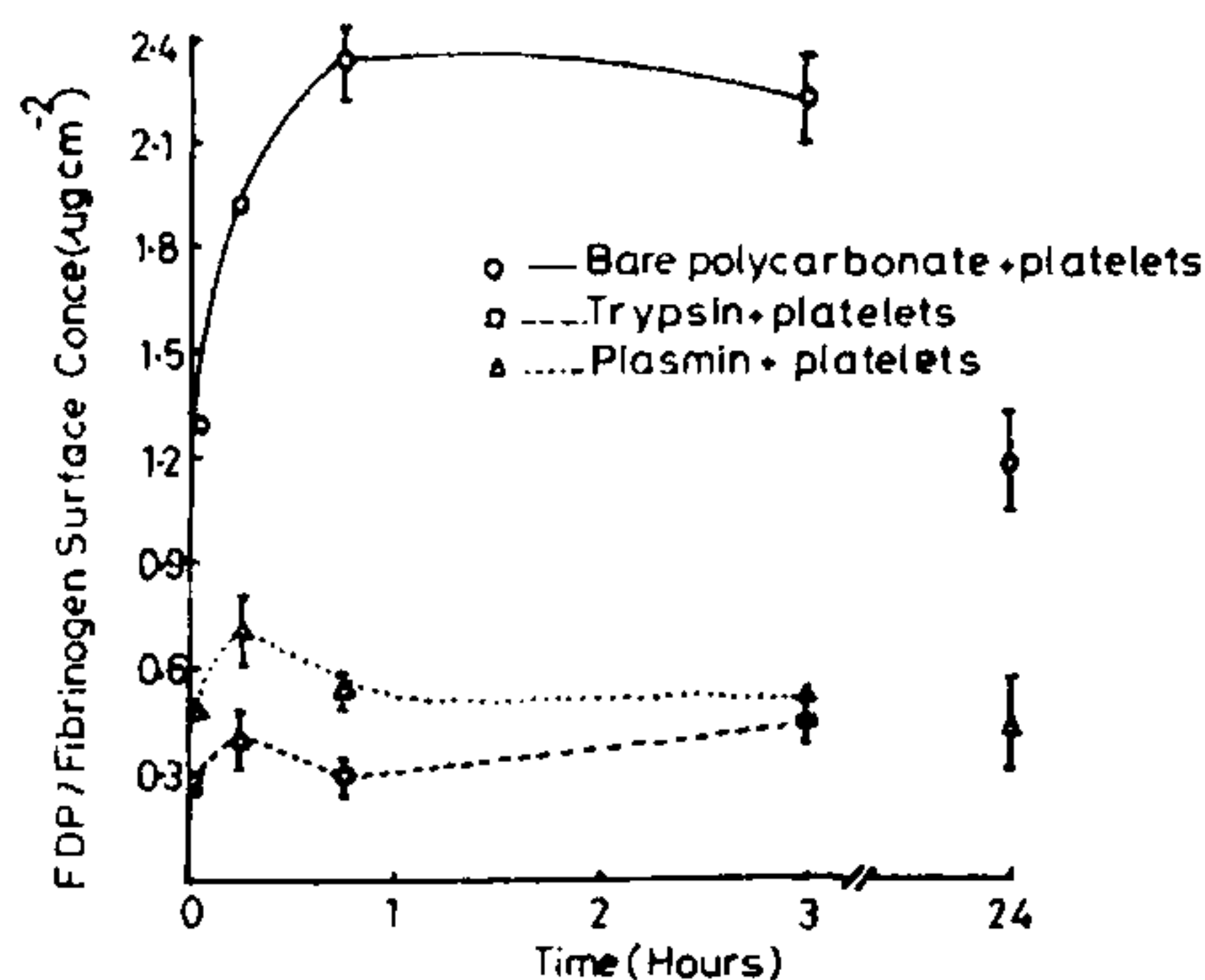


Figure 1. Adsorption of fibrinogen on polycarbonate as a function of time in presence of protease. Error limits are standard deviations from three experiments.

preadsorbed molecules are also desorbed off from the substrate (about 20-40%). Thrombin enhances the surface bound fibrin which has been extended with platelet-thrombin system as depicted in figure 3. The maximum fibrin-surface concentration reaches within one hour and gradually reduces with prolonged incubation time, which may be due to the detachment of the preadsorbed fibrin molecule. Figure 3 also dem-

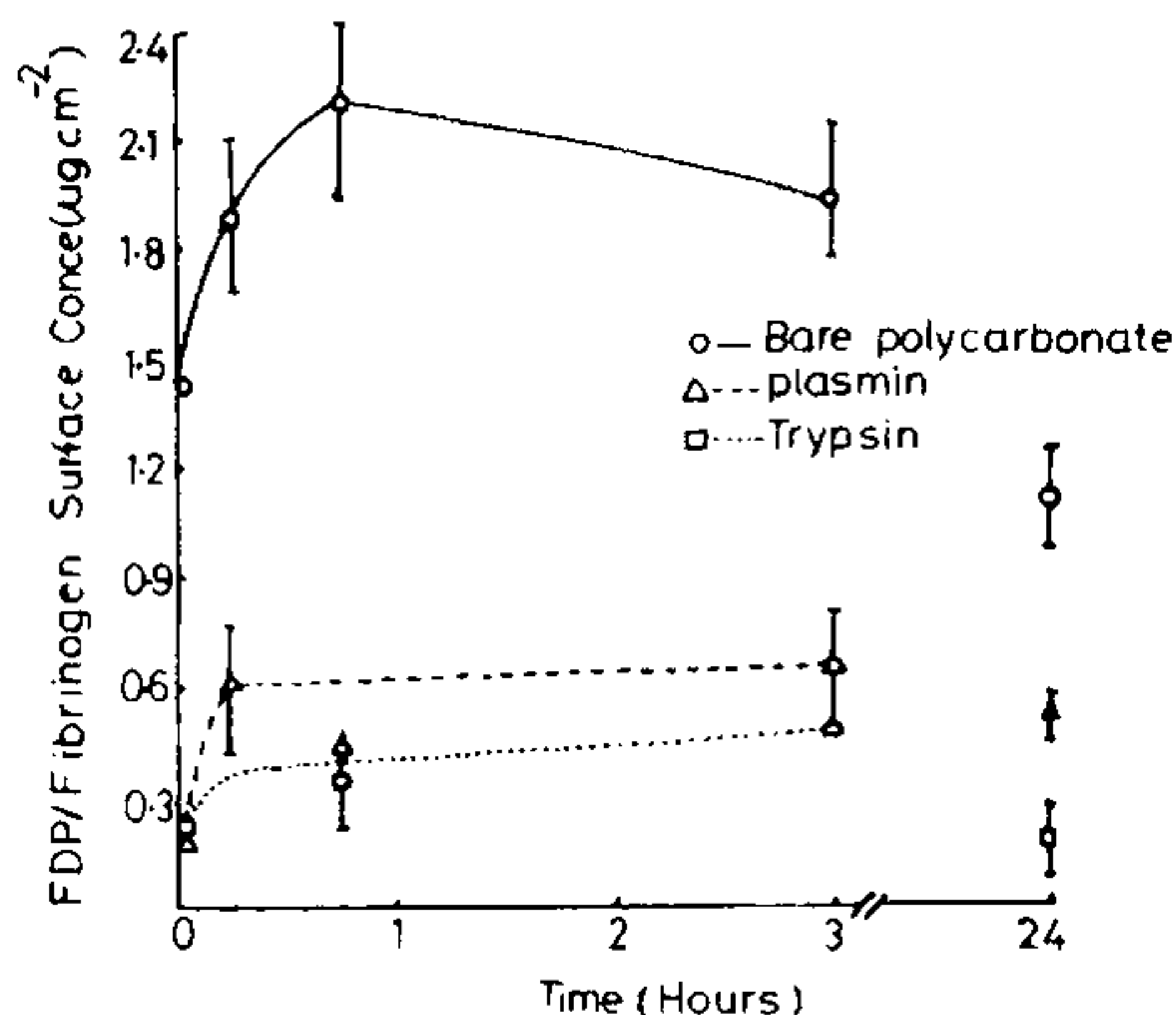


Figure 2. Adsorption of fibrinogen on polycarbonate as a function of time in presence of proteases and platelets. Error limits are standard deviations from three experiments.

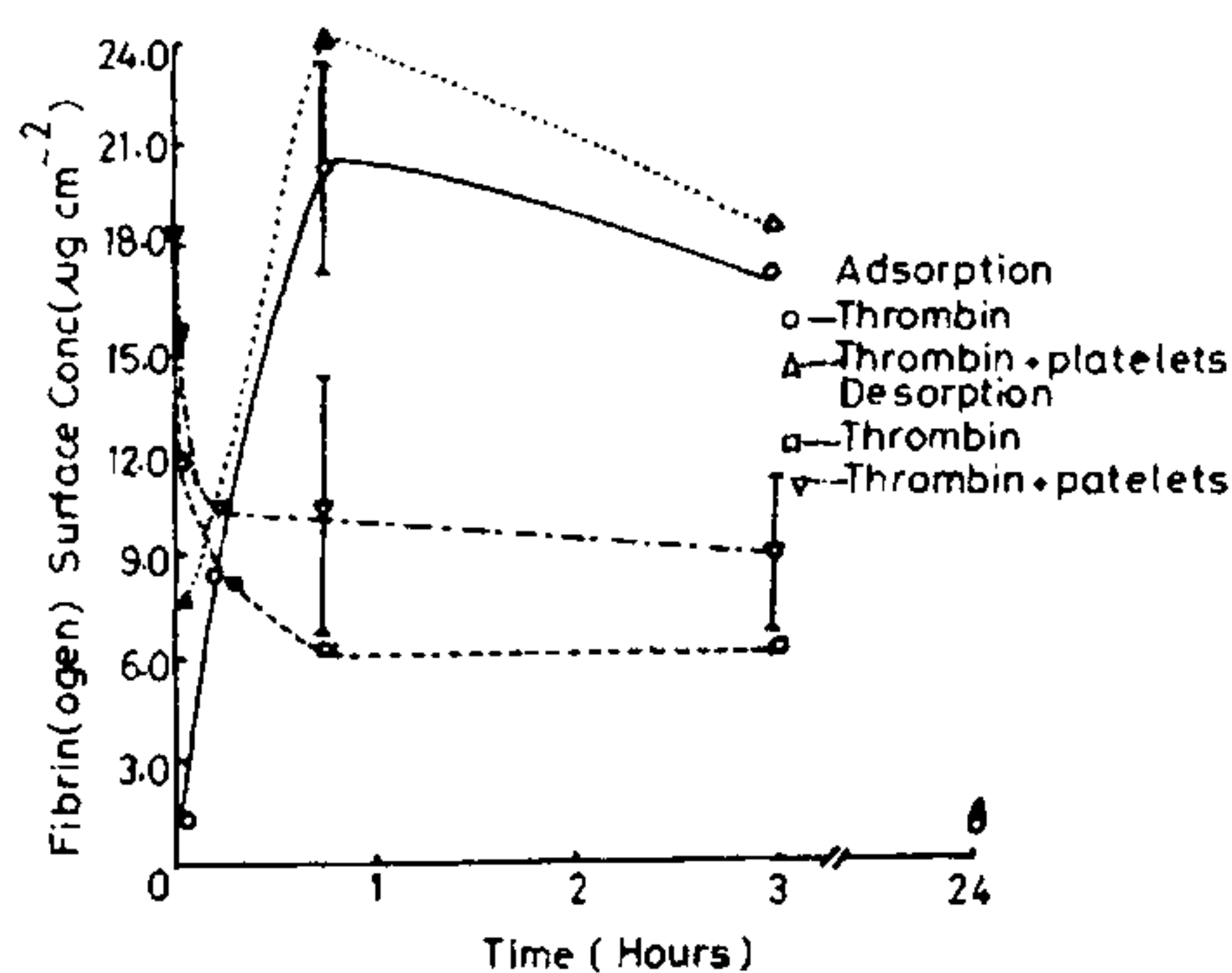


Figure 3. Adsorption and desorption of fibrin (ogen) on PC as a function of time in presence of thrombin and platelets. Error limits are standard deviations from three experiments.

onstrates that a part of the preadsorbed fg/fibrin is desorbed off from the surface. This may be due to the partial removal or exchange of surface bound fibrin-(ogen) with unlabelled species in the medium. It is obvious from table 1 that fg-induced platelet density on the surface is considerably inhibited by trypsin, but thrombin system has enhanced the effect. Thus from protein and platelet binding studies it is evident that the observed reduction in surface radioactivity with trypsin or plasmin system may be due to fibrinolysis of preadsorbed fg or less FDP (formed in solution) may be getting adsorbed to the substrate. However fibrin formed as a result of thrombin action on fg, has drastically deposited to PC surface, causing an enhanced surface level of it. It has been indicated that fibrin fibrils can augment platelet aggregation¹⁹, whereas FDP can inhibit platelet aggregation²⁰. Hence the present results agree with the surface fibrin-(ogen)/FDP concentration and platelet adhesion.

Figures 4 and 5 indicate that A α , B β and γ chains of fg bands are obtained on PC substrate eluted case (figure 4A), along with their degradation products in their solution form (figure 5A). Trypsin-treated fg system shows only one band in both cases (figures 4B and 5B), but more dense in their solution form. In case of thrombin-treated fg system, the fibrinopeptide is more adsorbed to PC substrate (figure 4C), though A α , B β , γ bands and degradation products (fibrinopeptides A and B) are also seen in solution. Brash *et al*²¹ also studied the plasmin effect on fg molecule on glass bead

Table 1 Concentration effect of trypsin and thrombin on FG induced platelet adhesion towards polycarbonate

Surfaces ^a	Mean Platelets \pm SD (Per mm ²) n = 25
1. Bare Polycarbonate (PC)	18.4 \pm 1.9
2. PC + 200 mg% Fibrinogen (fg)	43.0 \pm 4.0
3. PC + fg + 0.1 mg% Trypsin	40.2 \pm 3.8
4. PC + fg + 1 mg% Trypsin	38.4 \pm 3.5
5. PC + fg + 10 mg% Trypsin	35.2 \pm 3.7
6. PC + fg + 25 mg% Trypsin	30.7 \pm 4.6
7. PC + fg + 50 mg% Trypsin	28.4 \pm 5.2
8. PC + fg + 0.02 μ /ml Thrombin	48.8 \pm 4.4
9. PC + fg + 0.2 μ /ml Thrombin	49.6 \pm 3.8
10. PC + fg + 0.05 μ /ml Thrombin	55.0 \pm 6.0
11. PC + fg + 1 μ /ml Thrombin	55.2 \pm 6.0

^a Platelet suspension containing fibrinogen, trypsin and thrombin were exposed to PC (as demonstrated in surfaces 1 to 11).

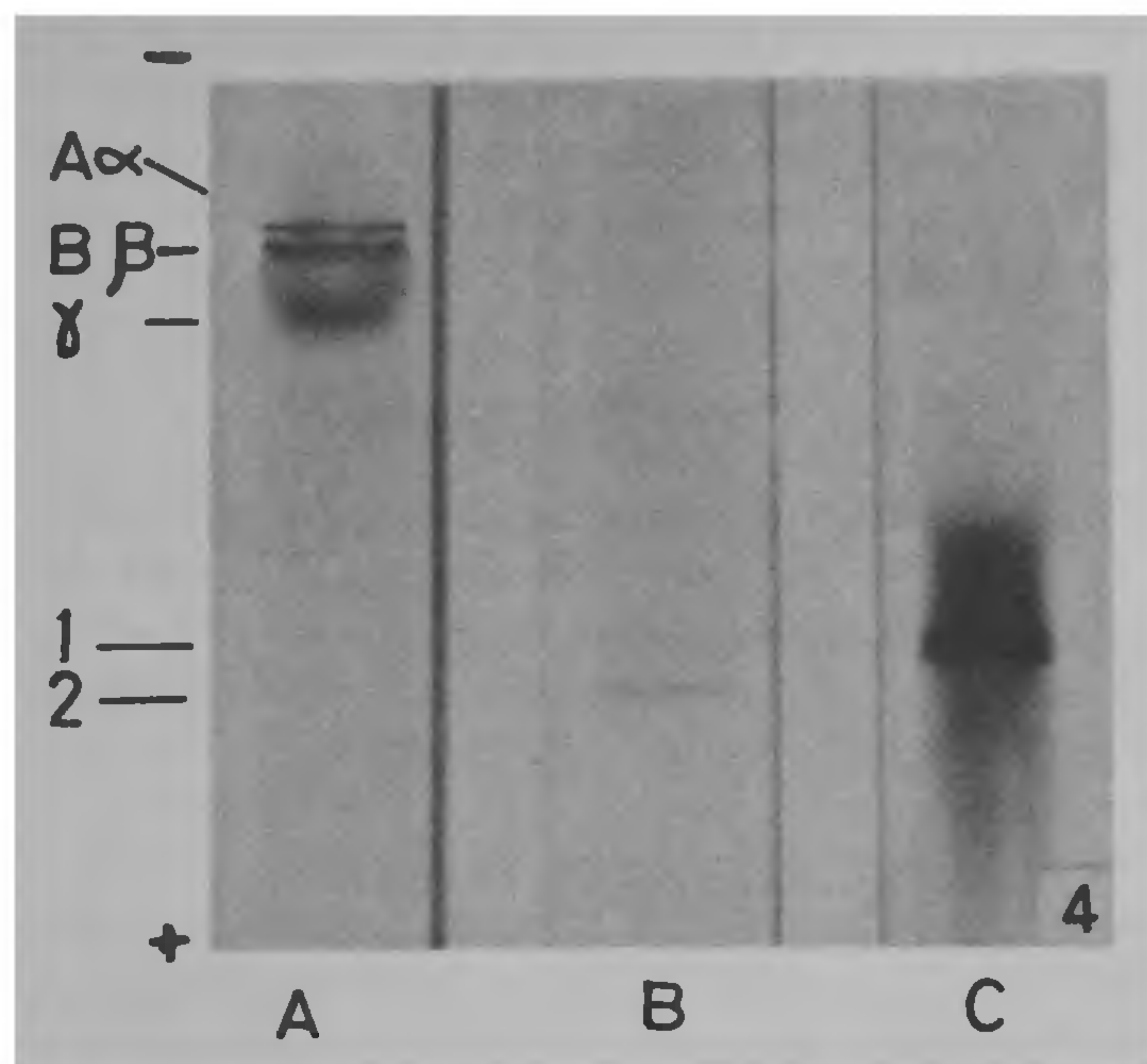


Figure 4. SDS-Polyacrylamide gel electrophoresis (reduced) of fibrinogen (A), trypsin-fibrinogen system (B), and thrombin-fibrinogen system (C) after elution from PC substrate (3 hours incubated to PC).

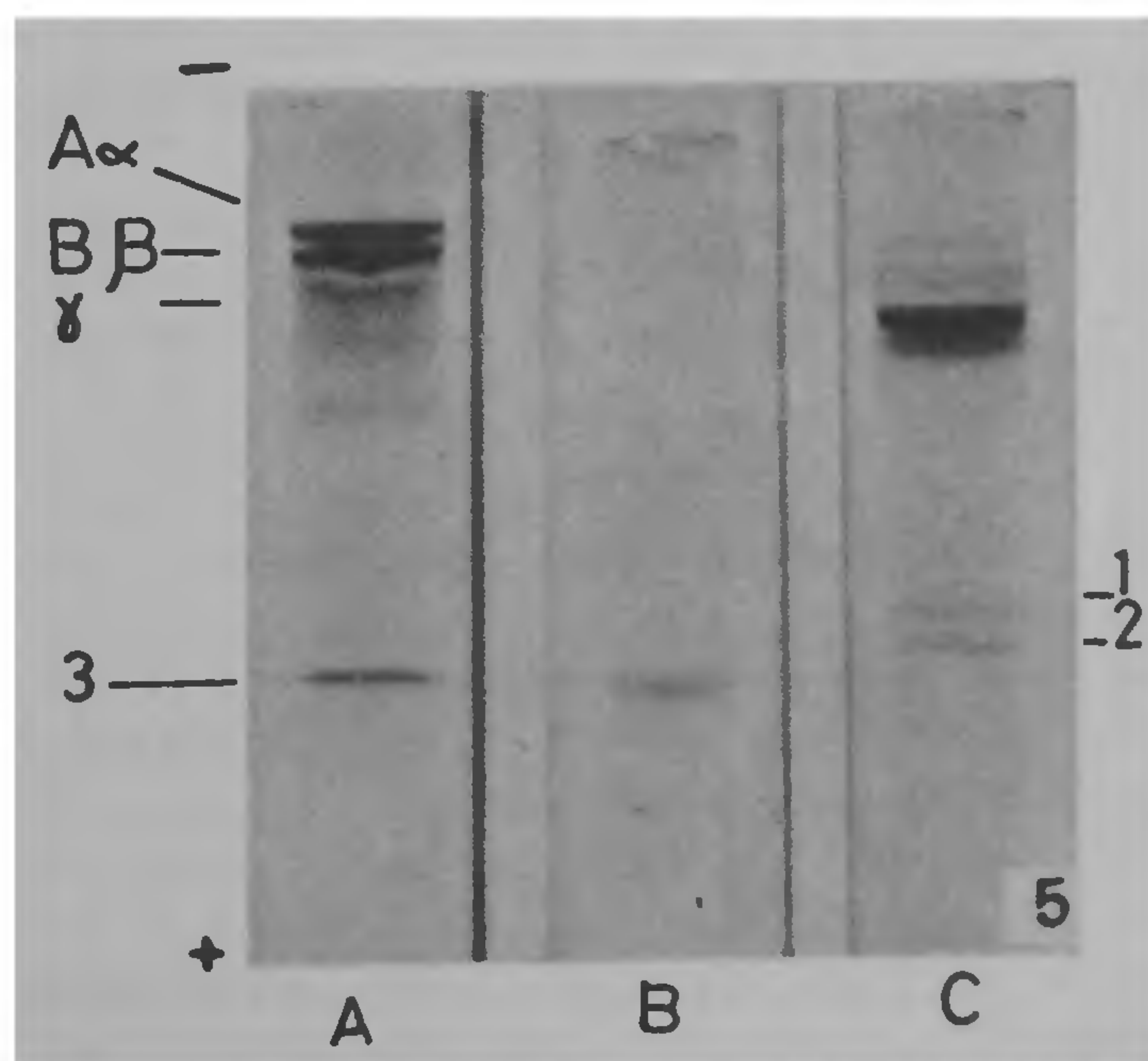


Figure 5. The same in their bulk solution (see results and discussion for details). 1,2 and 3 various fg degradation products.

columns and showed that plasmin degraded fg fragments are adsorbed to the substrate. Hence, it seems that several populations of molecules are formed in solution as a result of protease action, but the nature of the products adsorbed to the substrate may vary with

surface properties and the extent of protease activation by the substrate, resulting in changes in subsequent thrombotic process at the interface. Further studies are in progress to understand the initial phases of reactions, though more complex in nature.

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