Plasma Crosslinked Fibrin Polymers: Quantitation Based on Tissue Plasminogen Activator Conversion to D-dimer and Measurement in Normals and Patients With Acute Thrombotic Disorders

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Plasma crosslinked fibrin polymers (XLFP) are formed as a result of in vivo hemostatic activation and are elevated in thrombotic disease. We have investigated the plasmic degradation of plasma XLFP in vitro to provide information regarding the pattern of crosslinking and the composition of degradation products. Plasma XLFP were identified by sodium dodecyl sulfate (SDS)-agarose electrophoresis and Western blotting and quantitated by gel scanning. D-dimer was measured by enzyme-linked immunosorbent assay and the results were verified by SDS-polyacrylamide gel electrophoresis and Western blotting of the digests. Complete degradation of XLFP occurred only after supplementation of plasma with plasminogen (5 U/mL) and incubation with recombimant tissue plasminogen activator (rt-PA), indicating that the normal plasma plasminogen concentration limits plasma degradation in vitro. Gel electrophoresis showed that the principal terminal degradation products of XLFP were fragments D, DD, and E, indicating that crosslinking occurred primarily through γ chain dimers. After adding a low concentration of thrombin to plasma in vitro, XLFP increased progressively before clotting, and the concentration correlated with the increase in the D-dimer concentration after degradation (r = .98). Plasma XLFP and D-dimer concentrations in plasma digests were significantly elevated in patients with stroke (150 ± 83 μg/mL and 88 ± 32 μg/mL), myocardial infarction (217 ± 110 μg/mL and 84 ± 30 μg/mL), and venous thrombosis (187 ± 80 μg/mL and 86 ± 19 μg/mL compared with normals (28 ± 12 μg/mL and 25 ± 7 μg/mL). There was a strong correlation between the plasma concentration of XLFP and the D-dimer immunoreactivity of plasma after plasmic degradation (r = .87). The results indicate that XLFP in plasma are crosslinked primarily through γ chains and degrade to fragment DD with plasminogen activation. Also, the immunoreactivity of in vitro plasma digests of plasma reflects the concentration of XLFP and may provide a useful indirect measure of in vivo hemostatic activation in patients with thrombotic disease.

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HEMOSTATIC activation is primarily a localized process resulting in fibrin formation at sites of vessel injury, inflammation, or thrombus formation. However, the thrombin that is formed results in systemic effects, including release of fibrinopeptide A from fibrinogen and circulation of “soluble fibrin.” The latter is heterogeneous in composition and reflects a variable extent of polymerization and crosslinking. Low concentrations of soluble fibrin are found in normal plasma and increased concentrations are found in patients with thrombotic disease using several methods, including gel filtration chromatography,5 high performance liquid chromatography,6 sodium dodecyl sulfate (SDS)-agarose gel electrophoresis,8-9 potentiation of tissue plasminogen activator (t-PA) activity,10 ethanol gelation,12 and protamine sulfate precipitation.13

An alternative approach to the evaluation of plasma soluble fibrin is based on the identification of covalently crosslinked fibrin polymers (XLFP) resulting from the action of factor XIII4 to crosslink fibrin into dimers and polymers.14 Using electrophoretic techniques, we9 and others10-13 have identified low concentrations of XLFP in normal plasma and elevated concentrations in patients with thrombotic disease, including acute myocardial infarction (MI). Crosslinked fibrin polymers contain the γγ chain crosslink7,15,17-19 which is resistant to plasmin,20,21 and plasma degradation yields derivatives containing crosslinked γγ chain remnants, including fragment DD.21-24 In plasma of patients undergoing fibrinolytic therapy, plasma XLFP are degraded in vivo, contributing to elevated plasma concentrations of fibrin degradation products.25

In this study, we have investigated the plasmic degradation of XLFP in vitro. Conditions required for complete degradation of plasma XLFP are identified and relations between the concentration of XLFP and the D-dimer immunoreactivity after degradation are characterized. The results indicate that D-dimer is the primary plasmic derivative of XLFP and that the D-dimer concentration in plasma digests of plasma provides an indirect measure of the content of XLFP. Also, the plasma concentration of XLFP in normals and patients is measured, showing an increase in patients with thrombosis.

MATERIALS AND METHODS

Patients and blood samples. Blood was obtained by antecubital venipuncture from normals and patients, anticoagulated with sodium citrate (0.4% final concentration), immediately placed on ice, centrifuged within 1 hour of collection at 2,300 g for 15 minutes at 4°C, aliquoted, and stored at −70°C. Samples were thawed within 7 days and prepared for electrophoresis or plasmic digestion. Acute MI was diagnosed in patients with persistent chest pain, an elevated MB fraction of creatine kinase, and electrocardiographic changes of ST segment elevation with subsequent development of significant Q waves (transmural) or ST segment depression (subendocardial). Stroke was a clinical diagnosis based on the development of irreversible neurologic signs and symptoms and the exclusion of other disorders, such as tumors, infections, demyelinating diseases, and trauma, with confirmation by appropriate radio-

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graphic or magnetic resonance imaging. Deep vein thrombosis was diagnosed by venography in patients with compatible clinical findings.

**Fibrinogen and fibrin digests.** Fibrinogen (grade L) was purchased from Helena Laboratories (Beaumont, TX) and reconstituted at a concentration of 5 mg/mL in 50 mmol/L Tris, 100 mmol/L sodium chloride, pH 7.6. Plasmid digests were prepared by incubation with plasmin (kindly provided by the Bureau of Biologic Standards, Bethesda, MD) at a concentration of 0.15 Committee on Thrombolytic Agents (CTA) UlmL, at 37°C for 90 minutes. Digestion was terminated by the addition of aprotinin (Mobay Chemical Co, New York, NY) to a final concentration of 300 kallikrein inhibitory units (KIU)/mL to inhibit plasmin. Fragment DD was isolated from a plasmid digest of crosslinked fibrin prepared as described elsewhere. Lyophilized finely ground fibrin was digested by suspending in 50 mmol/L Tris, 100 mmol/L sodium chloride, 5 mmol/L calcium chloride, pH 7.6, and incubation with 3.4 CTA UlmL plasmin with gentle magnetic stirring at 37°C for 24 hours. Digestion was terminated by the addition of aprotinin (100 KIU/mL) to inhibit plasmin, and fragment DD was purified by gel filtration on a column (2.5 x 140 cm) of Sephaclyl S-300 (Pharmacia LKB Biotechnology, Inc, Piscataway, NJ) in 50 mmol/L Tris, 150 mmol/L sodium chloride, 40 mmol/L sodium ethylenediaminetetraacetic acid (EDTA), pH 7.6, at a flow rate of 35 mL/h. The protein peak containing fragment DD was identified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of aliquots, pooled, and stored.

**Preparation of plasma digests.** Aliquots of 50 µL of plasma were incubated with recombinant t-PA (rt-PA) purchased from Genentech, Inc (South San Francisco, CA) alone or with human plasminogen obtained from Sigma Chemical Co (St Louis, MO) at 37°C, and 500 KIU/mL aprotinin was added at intervals to inhibit plasmin. Normal plasma contains 1 U/mL plasminogen (2.4 µmol/L).

**Electrophoretic analysis.** SDS 2% agarose electrophoresis and SDS 4% to 10% gradient PAGE were performed as described elsewhere. Western blotting was performed using a modification of the method of Towbin et al as described previously. Immuno- staining was performed by incubating the nitrocellulose paper for 30 minutes at 25°C with the following sequence of antibodies and reagents: rabbit anti-human fibrinogen antiserum (Cappel Laboratories, Westchester, PA) diluted 1:2,000 in Tween tris-buffered saline (TTBS); biotinylated goat-antirabbit IgG (Bethesda Research Laboratories, Gaithersburg, MD) diluted 1:1,000 in TTBS; streptavidin-hors eradish peroxidase conjugate (Bethesda Research Laboratories) diluted 1:1,000 in TTBS. The blot was washed in TTBS after each step and was developed by submerging it in a solution of 20 mmol/L, 3,3’-diaminobenzidine tetrahydrochloride (Sigma) in TTBS containing 3.4 mmol/L nickel chloride and 0.009% hydrogen peroxide. The reaction was terminated after color development (5 to 10 minutes) by washing with water, and the blot was dried and photographed. For quantitation, gels were scanned, and protein in bands quantitated by comparison with a standard curve as described previously.

**D-dimer enzyme-linked immunosorbent assay (ELISA).** Crosslinked fibrin degradation products were measured with an ELISA (Dimertest; American Diagnostica, Greenwich, CT) using a monospecific antibody (DD/3B6) reactive with a site associated with the factor XIIIa-mediated γγ crosslink and a panspecific tag antibody (4D2) reactive with fibrin and fibrinogen degradation products. Precoated plates were used, and the results were calculated with a standard curve from 78 to 5,000 ng/mL of purified fragment DD provided by the manufacturer.

**Statistical analysis.** Comparison of means was performed using the two-tailed t-test. Variance is described as ±SD.

**RESULTS**

To determine the conditions for in vitro degradation of XLFP, plasma samples containing varying amounts of XLFP were incubated in vitro with rt-PA or rt-PA plus plasminogen and then analyzed by SDS-agarose electrophoresis and Western blotting (Fig 1). Incubation of plasma with a high concentration of rt-PA (200 µg/mL) resulted in incomplete degradation of fibrinogen and XLFP (lanes 2, 5, and 8). Bands consistent with fibrinogen degradation products X and Y in addition to fragment D were present after 1 hour of incubation with 200 µg/mL rt-PA in all samples, and less degraded derivatives larger than fibrinogen were evident in the digest in lane 5. Supplementation with 1 or 2 U/mL plasminogen resulted in greater, but still incomplete, degradation with rt-PA (data not shown). However, complete degradation of fibrinogen and XLFP occurred in all plasma samples after incubation for 1 hour with 25 µg/mL rt-PA plus 5 U/mL plasminogen (Fig 1, lanes 3, 6, and 9). After digestion under these conditions, electrophoresis

![Figure 1](https://example.com/figure1.png)
Fig 2. Formation and degradation of plasma XLFP. (A) Increasing XLFP in plasma after addition of thrombin. Stored, pooled citrated plasma was incubated with 0.01 U/mL thrombin (0.9 pmol/L) and 10 mmol/L calcium chloride at 37°C. Aliquots were withdrawn at intervals and subjected to SDS 2% agarose electrophoresis followed by Western blotting with antifibrinogen antiserum. The "Time/Clotting Time" value reflects the incubation time of each sample divided by the time of first visible fibrin formation at 35 minutes. (B) Plasmin digestion of plasma containing increasing amounts of XLFP formed by addition of thrombin. Aliquots of plasma were withdrawn from plasma samples shown in (A) and incubated with 25 µg/mL rt-PA and 5 U/mL plasminogen for 1 hour at 37°C. Aprotinin (500 U/mL) was added to each digest, and an aliquot was subjected to SDS 4% to 10% gradient PAGE, followed by Western blotting with antifibrinogen antiserum. The .57 time/clotting time value was not analyzed in this experiment.

showed no bands larger than fragment DD, a heavy band with the migration of fragment D, and a fainter band consistent with fragment DD. Because incubation of plasma with 25 µg/mL rt-PA plus 5 U/mL plasminogen resulted in apparently complete degradation, all digests in subsequent experiments were prepared using these conditions.

To determine whether a correlation existed between the D-dimer immunoreactivity of plasmin digests and the plasma concentration of XLFP, we prepared plasma containing varying concentrations of XLFP. A low concentration of thrombin (0.01 U/mL, 0.9 pmol/L) was added to stored citrated pooled plasma, resulting in a progressive increase in XLFP before visible clot formation occurred (Fig 2A). Whereas the monomer band did not change in intensity after addition of thrombin, the dimer band increased slightly at .14 and .28 of the clotting time, and was more prominent at .43 and .57 of the clotting time. At .71 and .86 of the clotting time, the dimer band was most prominent, with six to nine polymeric forms. The intensity of all bands decreased at 1.0 of the clotting time (35 minutes), when faint fibrin strands were first visible in the plasma and before solid clot formation occurred.

Incubation of aliquots of the same plasma samples with rt-PA and plasminogen yielded digests that contained predominantly fragments D, E, and DD by SDS-PAGE (Fig 2B). Whereas bands corresponding to fragments D and E did not change up to .86 of the clotting time, the D-dimer band increased progressively from .28 to .43 and was most
prominent at .71 and .86 of the clotting time, corresponding to the increase in XLFP (Fig 2A). The intensity of the E, D, and D-dimer bands decreased at the time of visible fiber formation (1.0 of the clotting time), in parallel with the decrease in the monomer and polymeric forms on the SDS-agarose gel. This correspondence was examined more closely by comparison of the plasma concentration (μg/mL) of XLFP as determined by SDS-agarose gel electrophoresis and scanning densitometry and as the ratio of polymer to monomer bands, with D-dimer concentration in plasmic digests assayed by ELISA (Fig 3A). Before addition of thrombin to the plasma, XLFP concentration, the ratio of polymer to monomer, and the digest DD concentration were 180 ± 52 μg/mL, .17 ± .02, and 44 ± 7 μg/mL, respectively. These values increased 1.3-, 1.2-, and 1.9-fold at .28 of the clotting time, 2.7-, 2-, and 3.1-fold at .57 of the clotting time, and to 7.4-, 5.8-, and 7-fold at .86 of the clotting time. Regression analysis indicated a high correlation between D-dimer immunoreactivity in the plasmic digests and XLFP expressed as concentration (r = .98) (Fig 3B) or as the ratio of polymer/monomer (r = .80). D-dimer concentrations in the plasmic digests were also determined from densitometric scanning of Western blots of SDS-polyacrylamide gels (Fig 2B) and quantitated by comparison with a standard curve of purified fragment DD. Similar amounts of immunoreactive D-dimer were found in the digest by ELISA and by densitometric analysis of the gels (r = .87) (Fig 4).

The D-dimer immunoreactivity in plasmic digests of plasma from 12 normals was distributed over a narrow range, with a mean ± SD of 25 ± 7 μg/mL (Fig 5). After collection, maintenance of the blood sample at 4°C for up to 8 hours or at room temperature for up to 4 hours before centrifugation and preparation of plasmic digests did not alter the results (data not shown). Similarly, storage of citrated plasma at –70°C for up to 2 weeks before digestion did not alter the results obtained by ELISA. The addition of hirudin (20 U/mL), a specific thrombin inhibitor, or iodoacetamide (10 mmol/L), an inhibitor of factor XIIIa to plasma before digestion did not affect results, indicating a negligible effect of hemostatic activation during plasmic digestion. The interassay and intraassay coefficients of variance were both 13% with normal plasma. The concentration of XLFP in plasma from normal individuals was lower than that in pooled plasma after prolonged storage (Figs 2 and 3), reflecting storage-induced changes in the latter.

Increased D-dimer immunoreactivity was found in plasmic digests of plasma from patients with thrombotic disorders (Fig 5). The mean ± SD values for 10 patients with stroke was 88 ± 32 μg/mL, for 14 patients with myocardial infarction 84 ± 30 μg/mL, and for six patients with venous thrombosis 86 ± 19 μg/mL. The values were distributed over a wide range of 32 to 144 μg/mL, but only 4 of the 30 samples from patients were within the range of normals, and the mean D-dimer concentration of the digested plasmas in each patient group was significantly higher than in normal plasma (P < .005).

![Fig 3. Measurement of XLFP in thrombin-treated plasma samples and D-dimer immunoreactivity of plasmic digests. (A) Thrombin (0.01 U/mL) and calcium chloride (10 mmol/L) were added to stored, pooled normal plasma, incubated at 37°C, and aliquots withdrawn at the indicated intervals as in Fig 2A. Samples were subjected to SDS 2% agarose electrophoresis and Western blotting with antifibrinogen antiserum, and the concentration of plasma fibrin polymer (○—○) was determined by gel scanning and comparison with a standard curve of purified fibrinogen at known concentrations. The ratio of polymer/monomer (x—x) was determined directly from the gel scan tracing. Plasmic digests of the same plasma aliquots were prepared and D-dimer immunoreactivity (●—●) of the digests was measured by ELISA. The values represent mean ± SD of three experiments. (B) Correlation between plasma concentration of XLFP and D-dimer immunoreactivity in plasmic digests. As determined by linear regression, the equation for the line is y = .23X + 23 (r = .98).](image-url)
Fig 4. Comparison of D-dimer concentration in plasmic digests of thrombin-treated plasma as measured by ELISA and gel analysis. Plasma was incubated with 0.01 U/mL thrombin and 10 mmol/L calcium chloride and aliquots were withdrawn at intervals up to the time of clot formation as in Fig 1. Digests were prepared and subjected to SDS 4% to 10% gradient PAGE and Western blotting with antifibrinogen antisera. D-dimer concentration in plasmic digests was measured by ELISA and by gel scanning in comparison with a standard curve of purified fragment DD. The values are derived from three experiments. The equation of the line as determined by linear regression is $y = 0.98X + 47$ ($r = 0.87$).

The plasma samples from normals and patients that were used to prepare plasmic digests were also evaluated by SDS-agarose electrophoresis and Western blotting (Fig 6A). Bands corresponding to XLFP were faint in normals, but were clearly visible, although to a variable extent, in the three patient groups. After plasmic degradation, prominent bands corresponding to fragments D and E were present by SDS-PAGE and were approximately the same in all samples (Fig 6B). A band corresponding to fragment D-dimer could not be clearly identified in plasma from normals, indicating that its concentration was less than 100 µg/mL, which is the limit of sensitivity of the electrophoretic method. The prominence of the fragment DD band by SDS-PAGE in patient samples corresponded to the intensity of XLFP in the same sample by SDS-agarose electrophoresis. For example, fibrin polymer bands were faint on the SDS-agarose gel in the patient with stroke in lane 4 of Fig 6A, and no clearly identifiable D-dimer band was present in the corresponding SDS-PAGE. In contrast, XLFP bands were prominent in the samples from a patient with stroke in lane 7 and with acute MI in lane 10, and the fragment DD band was easily identified in the corresponding digest samples (Fig 6B).

The concentration of XLFP and the polymer/monomer ratio in patient samples were significantly higher than in normals ($P < .005$) (Table 1). Similarly, the D-dimer immunoreactivity in plasmic digests of plasma from each of the three patient groups was significantly higher than in normals ($P < .005$). Just as was found for XLFP prepared in vitro (Fig 3), linear regression analysis showed a good correlation ($r = .87$) between the plasma concentration of XLFP and the D-dimer immunoreactivity of plasmic digests (Fig 7), with a best-fit equation of $y = 0.28X + 28$. D-dimer immunoreactivity in the digests measured by ELISA was closely correlated with D-dimer concentration determined by band intensity after SDS-PAGE (Fig 6B).

DISCUSSION

In the present study, we have shown that the D-dimer immunoreactivity of plasmic digests of plasma reflects the concentration of plasma XLFP. To obtain this result, complete degradation to fragment DD was necessary because less complete digests contain heterogeneous mixtures of larger crosslinked fibrin degradation products with lower immunoreactivity. Incubation of plasma with rt-PA at a high concentration (200 µg/mL) degraded XLFP incompletely, but the addition of supplemental plasminogen (5 U/mL) to plasma containing rt-PA at a lower concentration (25 µg/mL) resulted in complete degradation. This is consistent with a prior report showing incomplete degradation of fibrinogen and XLFP after incubation of plasma in vitro with plasminogen activator, and also with the demon-
Myocardial Deep Vein Normals Stroke Infarction Thrombosis

123 4567 8 9 10 11 12 13 14

Trimer – 16 22 28

Dimer – 80 160 150 280 120 170 220

Monomer – 320 220 260 310

Fibrin Polymer (µg/ml)

Normals Stroke Myocardial Infarction Deep Vein Thrombosis

1 2 3 4 5 6 7 8 9 10 11 12 13 14

DD by ELISA (µg/ml)

20 14 31 40 96 102 140 88 98 140 100 97 106

Table 1. Content of Plasma XLFP and of D-Dimer in Plastic Digests of Plasma From Normals and Patients With Thrombotic Disorders

<table>
<thead>
<tr>
<th>Plasma XLFP concentration (µg/mL)</th>
<th>Normals (n = 12)</th>
<th>Acute MI (n = 14)</th>
<th>Stroke (n = 10)</th>
<th>Venous Thrombosis (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>28 ± 12</td>
<td>217 ± 110</td>
<td>150 ± 83</td>
<td>187 ± 80</td>
<td></td>
</tr>
<tr>
<td>Plasma ratio of polymer/monomer</td>
<td>.16 ± .02</td>
<td>.30 ± .1</td>
<td>.28 ± .13</td>
<td>.32 ± .06</td>
</tr>
<tr>
<td>D-dimer concentration in plastic digests (µg/mL)</td>
<td>25 ± 7</td>
<td>84 ± 30</td>
<td>88 ± 32</td>
<td>86 ± 19</td>
</tr>
</tbody>
</table>

All values are mean ± SD.

r = .98 (Fig 3B). The reliability of measuring digest D-dimer by ELISA was confirmed by its significant correlation with D-dimer concentration estimated from Western blots after SDS-PAGE (r = .87) (Fig 4).

A similar approach was used to evaluate XLFP in plasma from normals and patients with thrombotic disorders. The amounts of XLFP and of plasma digest DD were increased
in patients compared with normals (Table 1), with a good correlation between XLFP determined by gel electrophoresis and plasmic digest DD measured by ELISA (Fig 7). The amount of fragment DD detected by Western blotting of plasma digests (Fig 6B) corresponded to the amount of XLFP in plasma by SDS-agarose electrophoresis. This is associated with D-dimer immunoreactivity after plasmic digestion of 25 μg/mL (Table 1). Linear regression of the correlation between plasma XLFP concentration and D-dimer immunoreactivity (Fig 7) derived the equation y = .28X + 28. An XLFP concentration of 28 μg/mL in normals predicts a digest D-dimer continuation of 36 μg/mL, close to the observed mean in normals of 25 μg/mL (Table 1). The slope of .28 indicates an increase of D-dimer immunoreactivity of 280 μg/mL for an increase of 1,000 μg/mL in plasma XLFP, and is close to the value of .29 predicted on the relative mass of the D-dimer portion of a fibrin dimer, which is the predominant polymeric species present before degradation. This analysis, together with the highly significant correlation between digest DD concentrations measured by ELISA and by SDS-PAGE densitometry, suggest that increases in D-dimer immunoreactivity in plasma digests accurately reflect elevated levels of XLFP and prove that the measurements by the 3B6/4D2 ELISA reflect the actual concentrations of D-dimer in the plasmic digests of plasma.

It has been suggested that the D-dimer immunoreactivity using the 3B6/4D2 assay can be falsely elevated in the presence of high concentrations of fragment D because the secondary antibody (4D2) reacts with both fibrin and fibrinogen degradation products. However, prior reports have found no reaction with the assay at fragment D concentration of up to 200 ng/mL, which far exceeds the concentration of fragment D in the sample after dilution. Further, a concentration of fibrinogen degradation products of 2,200 μg/mL gave a reaction using the assay of only 1,300 ng/mL. Therefore, even if there is some level of crossreactivity or any other effect of fibrinogen degradation products on the 3B6/4D2 ELISA, the contribution to the total would be small but could contribute to the positive Y intercept of the linear regression analysis.

The correlation between plasma XLFP and D-dimer immunoreactivity both in the in vitro model (Figs 2 and 3) and in normals and patients (Fig 7) indicates that the D-dimer level measured by ELISA after plasmic digestion may be a useful measure of the amount of XLFP in plasma from normals and patients with thrombotic disorders. Plasma soluble fibrin has been measured previously by several techniques, based on its unique physical and chemical properties. Musumeci et al, using gel filtration chromatography, and Edgar et al, using affinity chromatography, reported levels of 25 μg/mL and 27 μg/mL, respectively, in normals, values similar to our findings. Others have reported higher levels in normals of 120 to 184 μg/mL using gel filtration chromatography and lower levels of 3 to 7 μg/mL using affinity chromatography and chromogenic...
The different concentrations may be attributed to different molecular species of soluble fibrin measured using the various methods. Despite these differences, all methods identify an increase of between 3- and 10-fold in soluble fibrin in patients with thrombotic disease, similar to our findings (Table 1). However, most of these techniques are difficult to perform and impractical for routine clinical use. Our approach is based on the specificity of D-dimer as a marker for XLFP and the significant correlation between plasma XLFP and D-dimer immunoreactivity of plasmic digests. The results indicate that measurement of D-dimer immunoreactivity in plasmic digests may be useful in evaluation of hypercoagulable states by providing a simple and accurate measure of the plasma content of XLFP.

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