Stability Studies of Human Fibrinopeptide A as Measured by Radioimmunoassay (36613)

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Structural studies of human fibrinogen have revealed that the fibrinogen molecule has 3 pairs of polypeptide chains designated α, β and γ (1). Enzymatic conversion of fibrinogen to fibrin by thrombin action is associated with release of fibrinopeptides, principally A from the α chain and B from the β chain (2–4). These peptides have previously been studied for their amino acid sequence, electrophoretic mobility and cleavage kinetics from fibrinogen in human (1, 5–10) as well as in several mammalian and avian species (4, 7–13). Recently a new technique of measuring the A peptide by radioimmunoassay has been reported from this laboratory (14) and raises the possibility of measuring peptide levels in clinical syndromes associated with intravascular coagulation. In view of this possibility account must be taken of the findings of Teger-Nilsson (15) who documented the degradation of fibrinopeptides A and B in human serum and showed the splitting off of the N-terminal alanine, aspartic acid and serine in addition to C-terminal arginine from fibrinopeptide A (FPA). Because of the evidence for the existence of proteolysis of the A peptide in serum, stability studies of the peptide as measured by radioimmunoassay were made.

Materials and Methods. A solution containing fibrinopeptide A was prepared by adding 10 NIH units of bovine thrombin (Parke-Davis, Detroit) to 10 ml of human fibrinogen (Kabi, Grade L, Stockholm) (2 mg/ml, pH 7.5). The clot was allowed to stand for 4 hr at 25° and then filtered through ultrafiltation membrane XM50 (Amicon Corporation, Lexington, MA). The filtrate was assayed for A peptide activity and stored at −10°. Plasma anticoagulated with heparin (50 units/ml blood) was prepared as previously described (16). An ultrafiltrate of plasma was obtained by filtering 3 ml of normal heparinized plasma through ultrafiltration membrane XM50 at 25°. A single urine specimen (pH 5.2) from a healthy donor was used. Tris–saline buffer (0.05 *M* Tris, 0.1 *M* saline, pH 8.5) was used as a diluent. Radioimmunossay of fibrinopeptide A was made at pH 8.5 as previously described (14).

Fibrinopeptide A was incubated with plasma after mixing 0.4 ml of fibrinopeptide A solution with 3.6 ml of normal plasma and dividing the mixture into four samples of 1 ml each for incubation at different temperatures. Samples were obtained from each incubation mixture at zero time and again at 4 hr, 24 hr and 1 week intervals. All samples were stored at −30° until tested by radioimmunoassay. The stability of the peptide in plasma ultrafiltrate, urine and buffer was examined by substituting these solutions for plasma, incubating and testing as described for the plasma experiments. The effect of temperature on the stability of the peptide in plasma was tested by heating samples of peptide and plasma to 100, 80, 70 and 60° for 30 min, respectively, before incubation at 37°. At the end of 72 hr, samples were again obtained for radioimmunoassay of fibrinopeptide A.

Soybean trypsin inhibitor (SBTI) (K and K Laboratories, NY) was dissolved in Tris buffer. One milliliter of the incubation mixture (0.1 ml fibrinopeptide A and 0.9 ml plasma) contained SBTI in a final concentration of 0.01, 0.02, 0.04 and 0.15 mg/ml,

respectively. British Anti-Lewisite (BAL) (Dimercaprol USP, Hynson, Wescott and Dunning, Inc., Baltimore, MD) and crystalline disodiumethylenediaminetetraacetate (EDTA) (Fisher Scientific) were added to 1 ml peptide–plasma mixtures to achieve final concentrations of 10, 20 and 40 μmoles/ml in the respective mixtures. Di-isopropylfluorophosphate (DFP) solution in isopropyl alcohol was added to 1 ml samples of fibrinopeptide in plasma as described above. Epsilon amino caproic acid was added to 1 ml peptide–plasma mixtures in final concentrations of 3, 30, 100 and 300 μmoles/ml. Trasylol (FBA Pharmaceuticals, Inc., NY) was used in concentrations of 150, 500 and 1000 units/ml in the incubation mixture.

Results and Discussion. Stability of peptide in plasma. No appreciable deterioration of fibrinopeptide A activity was noticed at the end of 4 hr. However, loss of activity was distinct at 24 hr and 1 week intervals when the peptide was incubated in plasma at 4–37°. 55–60% of the peptide activity was lost at 24 hr and 85% was lost at 1 week incubation at 25 and 37° (Fig. 1A). No loss of activity was noticed when the peptide–plasma mixture was incubated at −10° or at pH 2.5 (see Fig. 1A). Prior tenfold dilution of the plasma with buffer (pH 8.5) resulted in greater stability. Progressive deterioration of the peptide activity is consistent with the enzymatic degradation by the enzymes amino- and carboxypeptidase as suggested by

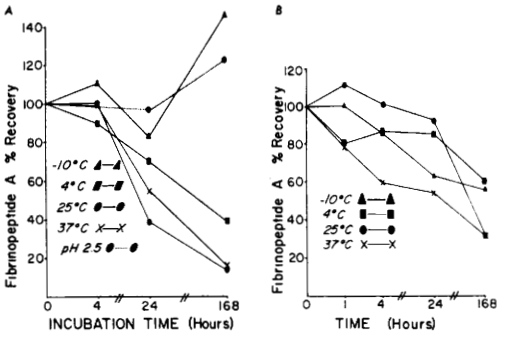

FIG. 1A. Fibrinopeptide A recovery (%) when incubated with normal plasma at different temperatures. (- - -) Results on incubation at pH 2.5. (B) Fibrinopeptide A recovery (%) when incubated with urine at different temperatures.

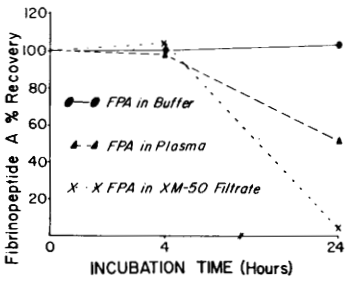

FIG. 2. Fibrinopeptide A recovery (%) when incubated with normal plasma, plasma XM50 ultrafiltrate and buffer (control) at 37°.

Teger-Nilsson (15). The enhanced stability of the peptide at pH 2.5 is also consistent with the enzymatic inactivity at the lower pH. Correlation of loss of immunoreactivity with the specific amino acids cleaved from fibrinopeptide A is yet to be made.

Stability in ultrafiltrate of plasma and in urine. Fibrinopeptide A immunoreactivity was unstable in urine (Fig. 1B). Loss of peptide activity was more rapid when incubated with a plasma ultrafiltrate as compared with incubation in whole plasma (Fig. 2). Only 5% activity was left at the end of 24 hr incubation. The presence of the presumed proteolytic activity both in plasma ultrafiltrate and urine suggests that the enzyme or enzymes responsible for the loss of peptide activity have a molecular weight less than 50,000 and are filterable through ultrafiltration and glomerular basement membranes.

Stability in buffer. In Tris–saline buffer pH 8.5, fibrinopeptide activity showed no change when incubated for up to 7 days at all the tested temperatures.

Effect of heating and proteinase inhibitors. Heating of the plasma–peptide mixture at 100, 80, and 70° for 30 min resulted in heavy protein precipitation. The stability was not improved when the incubation mixture was preheated at 60° for 30 min.

Of the various proteinase inhibitors tested (Table I) only EDTA in a final concentration of 40 μmoles/ml completely inhibited the loss of activity. EDTA itself in the concentrations tested did not affect the radioimmunassay. Though BAL by itself was not a significant inhibitor, when mixed with EDTA (BAL, 10 μmoles/ml and EDTA, 10 μmoles/

TABLE I. Effect of Proteinase Inhibitors on the Loss of FPA Activity in Plasma When Incubated at 37°.

Inhibitor		Inhibition (%)[a]	Inhibitor		Inhibition (%)[a]
Soybean trypsin inhibitor (mg/ml)	0.01	0	BAL, 10 μmoles/ml + EDTA, 10 μmoles/ml		100
	0.02	0			
	0.04	0	Di-isopropylfluorophosphate (μmoles/ml)	2.5	3
	0.15	0		5.0	3.5
BAL (Dimercaprol) (μmoles/ml)	10	3		7.5	3
	20	7	Epsilon amino caproic acid (EACA) (μmoles/ml)	3	6
	40	4		30	5
Ethylenediaminetetraacetate (EDTA) (μmoles/ml)	10	77		100	11
	20	72		300	45
	40	100	Trasylol (Aprotinin) (units/ml)	150	1
				500	1
				1000	0

[a] Percentage inhibition of the loss of FPA activity in plasma. In the presence of each inhibitor, 100% inhibition of FPA inactivation was considered to be present if the 72 hr sample had 2 μg/ml FPA activity in the incubation mixture. On the other hand, levels of 0.8 μg/ml were considered to be compatible with no inhibition of inactivation of the peptide provided by the inhibitor.

ml) 100% inhibition was noticed. The primary effect in the mixture is thought to be due to EDTA which selectively inhibits the aminopeptidases by chelating cations particularly Mg and/or Mn which are necessary for the enzyme activity of the aminopeptidases (17). Neither soybean trypsin inhibitor nor DFP in the concentrations tested made the peptide stable in plasma.

Conclusion. Stability studies of fibrinopeptide A in plasma, an ultrafiltrate of plasma, urine and buffer were conducted using a radioimmunoassay method to measure immunoreactivity of the peptide. The peptide was found to be unstable in plasma at 25 and 37° with 50 to 65% loss of activity at 24 hr and 85% loss of activity after 7 days. Similar loss of FPA activity was also noticed in an XM 50 ultrafiltrate of plasma and in urine. Of various proteolytic inhibitors tested only EDTA in concentrations of 40 μmoles/ml was able to completely stabilize the peptide against loss of activity. Freezing of the peptide in plasma at −10° was also associated with complete stability of FPA activity for at least 7 days.

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