

A THROMBIN ASSAY BASED UPON THE RELEASE OF FIBRINOPEPTIDE A
FROM FIBRINOGEN: DEFINITION OF A NEW THROMBIN UNIT

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(Received 13.4.1984; Accepted in original form 20.4.1984
by Editor L.J. Berliner)

ABSTRACT

An assay for thrombin is presented wherein thrombin-catalyzed hydrolysis at Arg-Au-16 to release fibrinopeptide A (FPA) from fibrinogen is measured using high-performance liquid chromatography (HPLC). In this assay one thrombin unit (TU) is defined as that amount of thrombin that will release half of the FPA in one min from one ml of a solution of >90% clottable normal human fibrinogen ($\leq 0.35 \mu\text{M}$) at 37°C, pH 7.4, $\Gamma/2$ 0.15. One TU is equivalent to ~ 0.1 NIH unit of thrombin and ~ 1 pmol of pure human thrombin. At 37°C, pH 7.4, and plasma levels of fibrinogen of 3 mg/ml, one TU will catalyze the release of 3.6 nmol FPA min^{-1} . Variability in fibrinogen samples which produce dramatic differences in clotting time assays with the same sample of thrombin, produce little or no variation in the catalytic assay for TU. The assay for TU obviates the need for maintenance of a thrombin reference standard.

INTRODUCTION

Thrombin is most frequently assayed from measurements of its effectiveness in clotting fibrinogen. In this assay, the time from the initiation of the reaction to the formation of a clot is related to the concentration of active thrombin. Several discussions and descriptions of the clotting time assay have been published (e.g. 1-6). Since the clotting time assay uses a natural substrate of thrombin, the assay should yield a reliable measure of the biological activity of a sample of thrombin. Clotting times, however, are functions of both thrombin-catalyzed proteolytic events and fibrinogen

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Key Words: Thrombin, Fibrinogen, Fibrinopeptide A

dependent polymerization reactions. It is therefore not surprising that thrombin clotting times vary with the quality of fibrinogen, the age of fibrinogen solutions, as well as with the presence of inhibitors of fibrin polymerization. This variability has made it necessary to maintain a thrombin reference standard. The commonly used NIH unit of thrombin is defined as that amount of thrombin which in 15 sec will clot 1 ml of fibrinogen that has been standardized with the thrombin reference standard. Thus, the activity of the thrombin sample is defined in terms of its potency relative to that of the thrombin reference standard rather than in terms of absolute catalytic activity. The need for the reference standard might be obviated by use of a thrombin assay based on direct measurement of its hydrolytic activity.

Assays based on direct measurements of the catalytic activity of thrombin should employ a specific substrate of thrombin (e.g. fibrinogen) to reflect the biological activity of the sample and to minimize the effect of other hydrolases which may be present in the sample of thrombin. In 1958, Jorpes et al. (3), proposed standardization of thrombin from measurements (at pH 9.0 and 20°C) of the appearance of N-terminal glycine, which occurs upon thrombin-catalyzed cleavage at the peptide bond between Arg-A₁₆ and Gly-A₁₇ in fibrinogen. The feasibility of this thrombin determination was demonstrated by Magnusson (4) in 1960. The assay which involves Edman degradation of the thrombin treated fibrinogen followed by separation and quantification of PTH-derivatives has not gained general acceptance. With the advent of HPLC and immunoassays for FPA, however, it has become more facile to monitor the thrombin mediated proteolysis at Arg-A₁₆. We present here an assay for thrombin which is based on measurements (using HPLC) of the rate of release of FPA which occurs in the thrombin catalyzed cleavage of fibrinogen at Arg-A₁₆. The assay obviates the need for a thrombin reference standard and yields a direct estimate of the proteolytic activity of thrombin at pH 7.4, 37°C, and $t_{1/2} = 0.15$.

METHODS

Fibrinogen - The laboratory prepared fibrinogen (L) was purified from outdated plasma by the method of Jakobsen and Kierulf (7), except that the fibrinogen solvent used to redissolve fibrinogen was the citrate saline solvent of Straughn and Wagner (8), and final dialysis was against 0.30 M NaCl. This fibrinogen preparation behaved identically to the fibrinogen preparation used previously in this laboratory (9). Other fibrinogen samples used were from commercial sources. Their source and designation (in parenthesis) in this paper are: Sigma #F-3879, Lot #81F-9365, (S-I); Sigma #F-4129, Lot #51F-9300 (S-II); Calbiochem #341576, Lot #201561 (C); Kabi, Grade L, Lot #30853 (K) and Cutter, Purengen^R, Code 611-68 (Cu). Weighed amounts of these powders were dissolved in 0.30 M NaCl, dialyzed, centrifuged and used. IMCO fibrinogen (I) in 50 mM Tris-HCl, 0.10 M NaCl, pH 7.5 was a generous gift from L. Lorand of the Department of Biology and Molecular and Cell Biology, Northwestern University, Evanston, Illinois, 60201. It was dialyzed into 0.30 M NaCl before use. All fibrinogen solutions were stored at -70°C prior to use.

Thrombin - Thrombin reference standard (Lot J) was provided by the Bureau of Biologics, Bethesda, MD, and contained 310 NIH units per ampule. Reconstitution was achieved either by addition of 1 ml of 0.01 M phosphate buffer, 0.14 M NaCl, pH 7.4, containing 0.5% PEG or by addition of 1 ml of standard assay buffer (see below). John W. Fenton II, New York State Department of Health, generously provided the purified human thrombin (2.87 NIH units/ μ g) used in this work. The thrombin which was greater than 93% active by active site titration (10) was stored in 0.3 M NaCl at -70°C. α -Thrombin accounted

for >99% of the thrombin in both samples. The concentration of thrombin was determined from its absorbance at 280 nm using an $E_{280}^{1\%}$ of 18.3 in 0.1 M NaOH and an M_r of 36,500 (11).

Clotting times - Clotting times were determined essentially as described by D.J. Baughman (5), wherein 0.2 ml of 1.58 mg/ml (clottable protein) was rapidly mixed with 0.1 ml of 3 NIH units/ml thrombin in a buffer system of 0.05 M phosphate, $\Gamma/2 = 0.15$, pH 7.0, 25°C giving a final concentration of 1.05 mg/ml clottable protein and 1 NIH unit/ml of thrombin. Clottable fibrinogen was determined by the method of Baughman (5). An $E_{280}^{1\%}$ of 15.1 and a molecular weight of 340,000 was used to calculate fibrinogen concentrations.

Reaction kinetics - The release of FPA was measured at pH 7.4, 37°C, in phosphate buffered solutions as specified in the text. In the recommended standard assay for release of fibrinopeptides, 1 ml of 3.5 μ M fibrinogen (7 μ M α -chains, 1.2 mg/ml) in 0.3 M NaCl was added to 10 ml of assay buffer. (The assay buffer was prepared by addition of a solution consisting of 0.05 M KH_2PO_4 , 0.1 M NaCl and 0.1% PEG 6000 to a solution consisting of 0.05 M Na_2HPO_4 and 0.1% PEG 6000 until pH 7.40 was obtained.) A 0.9 ml aliquot of the fibrinogen-containing assay buffer was transferred to a microfuge tube for determination (see below) of the peak area or height corresponding to FPA at zero time, $(\text{RFPA})_0$. Six ml of the remaining solution was then equilibrated at 37°C, and mixed with 10-200 μ l of an appropriate dilution of thrombin solution. Care was taken to dilute the thrombin stock solution with assay buffer containing PEG 6000 to prevent adsorption of thrombin on surfaces (12). Within 2 min of the addition of thrombin, before formation of a visible clot, 0.9 ml aliquots of the reaction mixture were added to six microfuge tubes which had been equilibrated at 37°C. To one tube, excess thrombin (e.g. 10 μ l of 3-5 NIH units/ml) was added for determination at the end of 30 min of the peak area or height corresponding to total releasable FPA, $(\text{RFPA})_f$. The remaining 5 tubes were incubated at 37°C, and quenched after the desired incubation times by addition of 0.1 ml of 3 M HClO_4 . After addition of the 3 M HClO_4 , which was also added to the zero-time and excess thrombin samples, water was added so that the total volume of liquid added to each sample was 1.3 ml. After 10 min the precipitated protein was removed by centrifugation, and 1 ml of the resulting supernatant solution was injected into the HPLC system for determination of the area or height of the FPA peak. An automatic sample injector (Wisp from Waters Associates) was used to facilitate handling of multiple samples. If sufficient points were not obtained at $\Delta \leq 0.25$ (see text) for a precise determination of TU/ml, additional values of Δ were determined with the remaining fibrinogen solution, using either other incubation times or another dilution of thrombin. The standard assay for TU/ml (see text) is independent of the absolute value of quenched assay solution injected into the HPLC system, provided that each determination of Δ is obtained from measurements of RFPA , $(\text{RFPA})_0$ and $(\text{RFPA})_f$ which are based upon the same amount of fibrinogen.

HPLC - A Varian 5000 liquid chromatograph was used for HPLC. The peptides were detected at 205 nm using a model 450 variable wave length detector from Waters Associates. Fibrinopeptide separation upon the 1 ml injection was achieved using a Waltman Particil-10-ODS-3 (0.46 x 25 cm), Ultrasphere OPS 5 μ (0.46 x 25 cm) or Spherisorb ODS-2 3 μ (0.46 x 10 cm) column, all equipped with a Waltman pre-column (0.21 x 5 cm). Various two-buffer gradient systems were studied for their effectiveness in peptide elution. The buffer systems were: from reference 6 where Buffer A is 0.083 M sodium phosphate (Fischer's HPLC grade H_3PO_4) adjusted to pH 3.1 with NaOH pellets and Buffer B is 6 volumes of

Buffer A mixed with 4 volumes of CH_3CN ; from reference 10 where Buffer A is 0.025 M ammonium acetate (adjusted to pH 6.0 with H_3PO_4) and Buffer B is 0.05 M ammonium acetate mixed 1:1 with CH_3CN ; and a buffer system described in a personal communication (from Drs. Ebert and Bell of The Department of Medicine at The Johns Hopkins University) where Buffer A is 0.1% $\text{CF}_3\text{CO}_2\text{H}$ (pH 2.0) and Buffer B is CH_3CN . The sodium phosphate system employed for the standard assay used a linear gradient system which went from 70%A, 30%B to 10%A, 90%B in 40 min at flow rate of 1 ml/min. The area under the FPA peak was proportional to the amount of FPA. Under conditions where sharp peaks were obtained (e.g., with the Spherisorb, ODS-2 3 μ column) the peak height was also proportional to the amount of FPA.

RESULTS

Several systems have been reported recently for the quantification of the release of fibrinopeptides from fibrinogen using reverse phase HPLC chromatography (e.g. 9,13,14). These systems which differ with respect to the column used and the composition of the aqueous component of the eluent buffer, were evaluated for their suitability for use in a thrombin assay. The composition of eluent buffer was found to be an important determinant of peptide recovery. The phosphate buffer system described here reproducibly yielded the highest recovery of FPA (>90%). Fig 1 depicts an HPLC chromatogram for 1 ml of

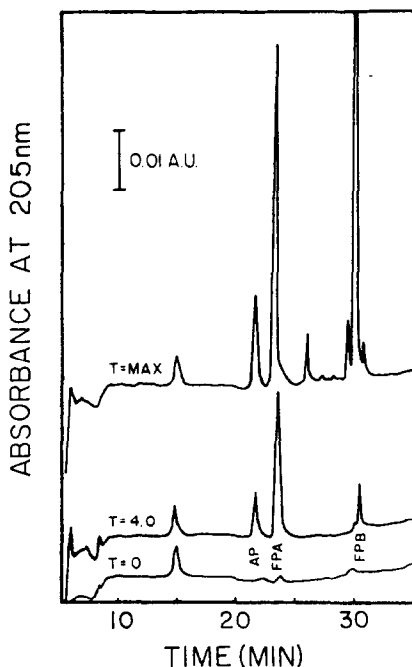


FIG. 1
HPLC elution profile of the fibrinopeptides from a kinetic run using Kabi Fibrinogen at 0.35 μM . Aliquots (0.9 ml) of reaction mixtures containing 0.02 NIH unit/ml thrombin were quenched at times 0 and 4.0 min. Another aliquot (T=Max) had 0.05 NIH unit/ml and was quenched at 30 min. HPLC conditions were those described for the 0.083 M phosphate pH 3.1 system using a 3 μ (0.46 x 10 cm) Spherisorb column. A peak corresponding to the void volume (not shown) is present in the first 5 minutes of the profile.

quenched assay mixtures containing 0.24 nmol fibrinogen before addition of thrombin (T=0), 4 min after the addition of 0.02 NIH unit of thrombin (T=4), and 30 min after the addition of 0.05 NIH unit of thrombin (T=Max). The peaks labeled AP, FPA and FPB have been identified as phosphorylated (on Ser-3) FPA, FPA and FPB, by their amino acid composition. Fibrinopeptide A and B have been reported (9) to yield peak areas (at 205 nm in a 1 cm cell) of 0.044 and 0.0512 absorbance units-ml/nmol, which correspond to molar absorptivities

of $4.4 \times 10^4 \text{ cm}^{-1}\text{M}^{-1}$ (FPA) and $5.12 \times 10^4 \text{ cm}^{-1}\text{M}^{-1}$ (FPB).

The thrombin-catalyzed release of FPA from fibrinogen has also been shown (15) to follow simple Michaelis-Menten kinetics. At pH 7.4, 37°C, $\Gamma/2 = 0.15$, K_m for the release of FPA is $7.2 \mu\text{M}$ based on the concentration of A α -chains. When the concentration of A α -chains is less than $0.1 \cdot K_m$ the Michaelis-Menten equation for FPA release

$$\frac{d[\text{FPA}]}{dt} = - \frac{d[\text{A}\alpha]}{dt} = \frac{k_{\text{cat}} [\text{Throm}]}{\frac{K_m}{[\text{A}\alpha]} + 1}, \quad (1)$$

reduces to

$$\frac{d[\text{FPA}]}{dt} = - \frac{d[\text{A}\alpha]}{dt} = \frac{k_{\text{cat}}}{K_m} [\text{A}\alpha] [\text{Throm}] \quad (2)$$

Integration of equation 2 yields

$$\ln \frac{[\text{A}\alpha]}{[\text{A}\alpha]_0} = - \frac{k_{\text{cat}}}{K_m} [\text{Throm}] \cdot t \quad (3)$$

Thus a plot of $\ln([\text{A}\alpha]/[\text{A}\alpha]_0)$ versus time should be linear with a slope proportional to the thrombin concentration, where $[\text{A}\alpha]_0$ and $[\text{A}\alpha]$ are the concentrations of intact A α -chains at $t=0$ and $t=t$, respectively. The ratio $[\text{A}\alpha]/[\text{A}\alpha]_0$ may be evaluated from the equation

$$\frac{[\text{A}\alpha]}{[\text{A}\alpha]_0} = \frac{[\text{FPA}]_f - [\text{FPA}]_0}{[\text{FPA}]_f - [\text{FPA}]_0} = \frac{(\text{RFPA})_f - (\text{RFPA})_0}{(\text{RFPA})_f - (\text{RFPA})_0} = \Delta, \quad (4)$$

where RFPA is the area or height of the FPA peak and the subscripts 0, and f denote values before the addition of thrombin and after sufficient incubation with thrombin so as to release all of the FPA. Peak height rather than area could be used for RFPA when the chromatogram yielded sharp peaks whose height was proportional to the amount of FPA.

A time dependence for the release of FPA is shown in Fig 2A for three samples of fibrinogen. The plot in Fig 2B illustrates the expected linear dependence of $\ln([\text{A}\alpha]/[\text{A}\alpha]_0)$ obtained for the fibrinogen samples. Substitution of $[\text{A}\alpha]/[\text{A}\alpha]_0 = 0.5$ at $t_{0.5}$ (one half-life for A α -chains) in equation 3 yields

$$\frac{1}{t_{0.5}} = \frac{k_{\text{cat}}}{K_m \ln 2} [\text{Throm}] \quad (5)$$

Since $1/t_{0.5}$ is proportional to the thrombin concentration, it may be used to specify thrombin content. If a thrombin unit (TU) is defined as that amount of human thrombin which will release half the FPA in one min from 1 ml of 90% clottable human fibrinogen ($\leq 0.35 \mu\text{M}$) at 37°C, pH 7.4, $\Gamma/2 = 0.15$; $1/t_{0.5}$ is equivalent to the concentration of thrombin units (TU/ml) in the assay mixture.

Inspection of equations 1-5 reveals that thrombin units can be determined from measurements of peak areas or height without knowledge of the absolute concentration of functional fibrinogen as long as the fibrinogen concentration is $\leq 0.35 \mu\text{M}$ (i.e. $\leq 0.70 \mu\text{M}$ A α -chains). The dashed lines in Fig 2B illustrate a graphical determination of $t_{0.5}$. Since the y- intercept of the line of Fig 2B should be one, the concentration of thrombin units may be determined from

the value of RFPA at a single time (preferably a time that gives a Δ -value close to 0.5) using a fibrinogen solution with a previously determined value of $(RFPA)_o$ and $(RFPA)_f$. Precise values of TU, however, are best determined using more than one value of Δ . It is also important to periodically reevaluate $(RFPA)_o$ and $(RFPA)_f$, since variations in flow rate, injection volume and fibrinogen concentration in the sample will cause the absolute values of $(RFPA)_o$, $(RFPA)_f$ and $(RFPA)$ to change.

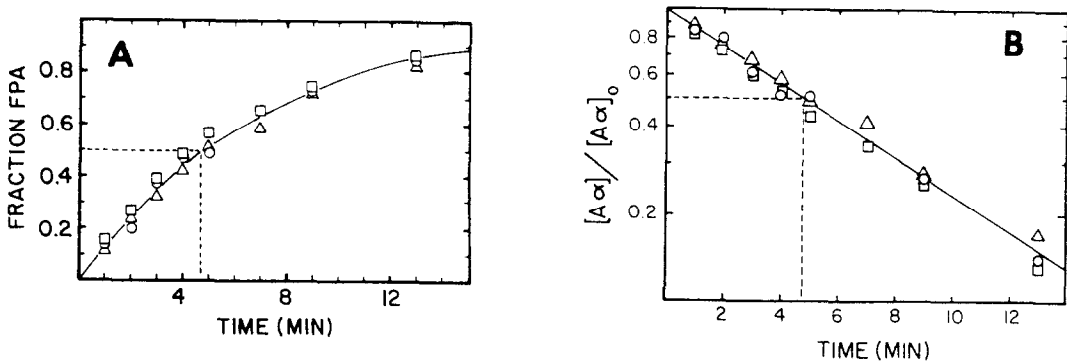


FIG. 2

Thrombin-mediated release of FPA from different samples of $0.35 \mu\text{M}$ fibrinogen (L), \odot ; (K), \square ; and S-I, \triangle ; at 10 mM phosphate, pH 7.4, 37°C , $\Gamma/2 = 0.15$, 0.02 NIH unit/ml thrombin. The plot in Fig 2A depicts the time course of the appearance of FPA, and that in Fig 2B depicts the first order decay of intact $A\alpha$ -chains. The solid lines were calculated using equation 3 and a value of 0.146 min^{-1} for $k_{\text{cat}} [\text{Throm}]/K_m$ which gave the best fit of the data to equation 3.

Thrombin units may also be estimated from equation 6.

$$\text{TU/ml} = 1/t_{0.5} = -\ln \frac{(RFPA)_f - (RFPA)}{(RFPA)_f - (RFPA)_o} / t \cdot \ln 2 = -(\ln \Delta) / t \cdot \ln 2 \quad (6)$$

Values of TU obtained by averaging several values of TU, estimated from measurements of Δ at times $\leq 2t_{0.5}$, should yield the most reliable estimates of TU. Values of Δ at $t > 2t_{0.5}$ should be avoided, however, since small errors in the determination of RFPA for these points can result in large relative errors in TU.

The data in Table I illustrate that the determination of TU is essentially independent of the fibrinogen sample used. Seven different samples of fibrinogen with clottabilities ranging from 79% - 96% exhibited $0.21 \pm 0.01 \text{ TU}$ when assayed with 0.02 NIH unit of thrombin having a specific activity of $2.87 \text{ NIH unit}/\mu\text{g}$. The variation between the highest and lowest determination of TU was 17%, whereas the corresponding variation for thrombin clotting times (determined with 1 NIH unit/ml of thrombin) was 140%.

Under the conditions used for the assay, the release of FPA is followed by end to end polymerization which in turn is followed by the release of FPB (15). It is therefore not surprising that the release of FPA is not affected

TABLE I

Determination of TU using Samples of Fibrinogen which Exhibit Different Clotting Times^a

FGN SAMPLE	TU/ml	% Clottable	Clotting Time (Sec)
L	0.209	96	39
S-I	0.209	94	46
S-II	0.224	79	34
C	0.209	91	43
I	0.212	89	54
Cu	0.191	89	22
K	0.208	94	34

^aTU/ml was evaluated with 0.32 μ M fibrinogen in 10 mM phosphate which was mixed under the assay conditions with 0.02 NIH unit of thrombin/ml. Clotting times were determined at 1 NIH unit/ml of thrombin.

by the presence of polymerization inhibitors such as Gly-Pro-Arg-Pro and EDTA (Fig 3). These inhibitors, however, drastically prolong the clotting times and inhibit the release of FPB (15). In addition to being insensitive to the presence of polymerization inhibitors the determination of thrombin units was unaffected by the presence of 1mM Ca^{+2} . The assays described up to this point in the text were carried out in the presence of a low concentration of phosphate buffer (10 mM) to approximate plasma concentrations of phosphate and

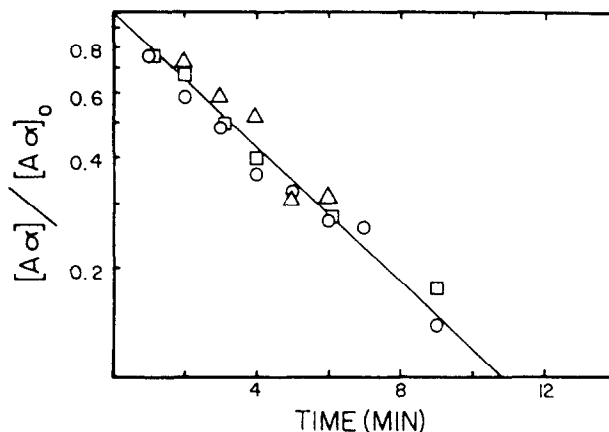


FIG. 3

Effect of EDTA and Gly-Pro-Arg-Pro on the release of FPA from fibrinogen (0.32 - 0.34 μ M) by thrombin (0.030 NIH unit/ μ ml) in 10 mM phosphate at pH 7.4, 37°C, $\Gamma/2 = 0.15$, 0.001% PEG. \circ , no additions; \square , 0.54 mM Gly-Pro-Arg-Pro; \triangle , 25 mM EDTA.

minimize precipitation which occurs when Ca^{+2} is added to a more concentrated phosphate buffer. To minimize the possibility of inadvertent alterations in pH a more concentrated phosphate buffer (0.05 M) is suggested for the standard assay. Fig 4 illustrates determination of TU concentration for four different

concentrations of a freshly reconstituted thrombin reference standard. The slope of the plot in Fig 4B indicates that in the recommended standard assay 1 TU is equivalent to 0.118 NIH unit. The estimate that 3.09 ± 0.56 NIH unit (11) is equivalent to 1 μg of pure human thrombin together with the observation that 1 TU is equivalent to 0.118 NIH unit indicates that 1 TU is equivalent to 1.05 ± 0.19 pmol of pure human thrombin ($M_r = 36,500$).

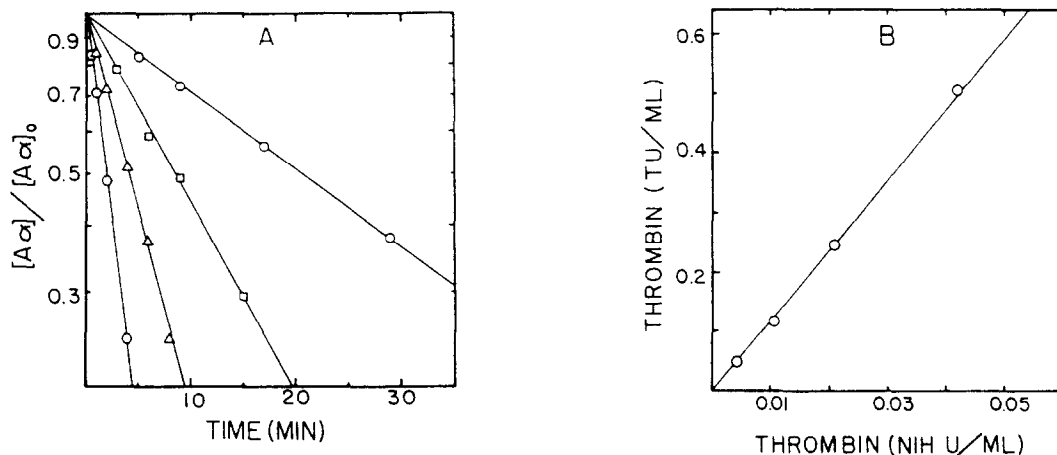


FIG. 4

Relationship between thrombin units and NIH units. The recommended standard assay described under "Methods" was used wherein 10 μl (\odot), 25 μl (\square), 50 μl (\triangle) and 100 μl (\diamond) of a stock solution of thrombin NIH reference standard containing 2.55 NIH units/ml was added to 6 ml aliquots of 0.32 μM fibrinogen in assay buffer to yield 0.00424, 0.0106, 0.0211, 0.0418 NIH unit/ml thrombin. The values of TU/ml ($1/t_{0.5}$) determined from plots in Fig 4A are plotted in Fig 4B against the corresponding values of NIH/ml in the assay buffer.

DISCUSSION

The standard assay for thrombin units described under "Methods" is based upon the proteolytic activity of thrombin toward one of its natural substrates under conditions of pH, temperature, and ionic strength close to those used in many biological applications of thrombin. The assay which is based upon thrombin catalyzed release of FPA is insensitive to the presence of polymerization inhibitors. It should also be unaffected by variations in fibrinogen structure spatially remote from the scissile bond. When normal human fibrinogen with a clottability $\geq 90\%$ is used, no significant dependence of the assay on the fibrinogen is detected. Thus, the assay does not require a thrombin reference standard for calibration. Another advantage of the assay is that the thrombin unit specifies the catalytic potency of thrombin samples in molecular terms. Additionally, rearrangement of equation 6 yields the relationship

$$[\text{TU}] \cdot K_M \cdot \ln 2 = k_{\text{cat}} [\text{Throm}] \quad (7)$$

where [TU] represents the concentration of thrombin units (TU/ml). Since $K_M = 7.2 \mu\text{M}$ under the assay conditions (15),

$$5.0 [\text{TU}] = k_{\text{cat}} [\text{Throm}] \quad (8)$$

Substitution of equation 8 in equation 1 yields equation 9

$$\frac{d[\text{FPA}]}{dt} = \frac{5.0[\text{TU}]}{\frac{7.2}{[\text{A}\alpha]} + 1} \quad (9)$$

which predicts the rate of release of FPA in $\mu\text{M}\cdot\text{min}^{-1}$ as a function of the μ molar concentration of $\text{A}\alpha$ -chains of fibrinogen and the concentration of thrombin. At a plasma concentration of fibrinogen of 3 mg/ml (17.6 μM $\text{A}\alpha$ -chains), one TU should catalyze the release of 3.6 nmol FPA/min at 37°C, pH 7.4.

The assay presented here differs from most determinations of enzyme activity in that it relies on the determination of half-lives for the first-order decay of substrate rather than the determination of initial velocities for substrate decomposition. Reproducible determination of initial velocities usually requires duplication of functional substrate concentrations. This could be problematical when different samples of fibrinogen are used. Half-lives for the first order release of FPA, however, should be independent of the initial substrate concentration, as long as $[\text{A}\alpha] \leq 0.1 \cdot \text{K}_m$. It is realized that the dependence of an initial velocity assay on variations in substrate concentration could be reduced by use of an initial substrate concentration which is substantially greater than K_m . It was not considered advisable, however, to design an assay with a high fibrinogen concentration, since the presence of competitive inhibitors (e.g. fibrinogen degradation products) which might be associated with certain fibrinogen samples could result in variations in initial velocity. That is, at a near saturating substrate concentration only a small fraction of the thrombin will be free, and the thrombin will be distributed between substrate and inhibitor according to their relative concentration and affinity for thrombin. At the low concentrations of fibrinogen specified for the assay presented in this work, less than 10% of the thrombin is saturated by fibrinogen. Under such conditions, contaminating competitive inhibitors are not likely to result in displacement of fibrinogen from thrombin.

It is important to note that in some chromatographic systems the phosphorylated FPA is not resolved from FPA. The time dependence of the release of these two peptides do not differ significantly at the low fibrinogen concentration used for the assay. Thus, thrombin units can be accurately determined from measurements of the areas or heights of peaks resulting from the combined presence of both peptides.

It is conceivable that certain laboratories will find it more convenient to continue to determine thrombin units from clotting times using a thrombin reference standard. The methods presented here, however, should also facilitate this approach by providing the means for direct measurement of the catalytic potency of the thrombin standard needed to calibrate the fibrinogen. Additionally, the observation that there are 8.5 TU/NIH unit establishes a relationship between the proteolytic activity and the clotting-time assay for human thrombin.

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