SENSITIVE FLUORESCENT DETERMINATION OF TRYPSIN-LIKE PROTEASES

Frances Brown, Michael L. Freedman and Walter Troll

New York University School of Medicine
Departments of Medicine and Environmental Medicine
New York, New York 10016

Received May 7, 1973

SUMMARY

A new sensitive assay for trypsin-like proteases has been developed using as substrate protamine sulfate from herring with the amino terminal group blocked with dinitrofluorobenzene. Enzymatic hydrolysis liberated amino groups which were quantitated by measuring fluorescence after reaction with fluorescamine. Thrombin was capable of hydrolyzing this substrate at a concentration as low as 8.3 NIH units per ml. Amino acid analysis of the protamine suggests that thrombin is capable of hydrolyzing a peptide bond other than an arginyl-glycine bond. Inhibition of thrombin by n-acetylimidazole suggests a relationship between the clotting and proteolytic activities of thrombin.

INTRODUCTION

Many proteolytic enzymes resemble trypsin in that they hydrolyze peptides at the basic amino acid sites, either arginine or lysine (1). Protamine would appear to be an ideal substrate for this type of enzyme as it is stable, soluble in water and contains 67% arginine (2). In the present study we describe a new assay for these trypsin-like enzymes using protamine with the amino terminal group blocked with dinitrofluorobenzene (DNFB). Enzymatic hydrolysis liberated amino groups which were quantitated by measuring fluorescence after reaction with fluorescamine, a new reagent for the detection of primary amines (3). This assay is capable of measuring nanogram concentrations of trypsin within twenty minutes.

Using this assay we have demonstrated that thrombin, a limited proteolytic agent (4), is capable of hydrolyzing a peptide bond in protamine.

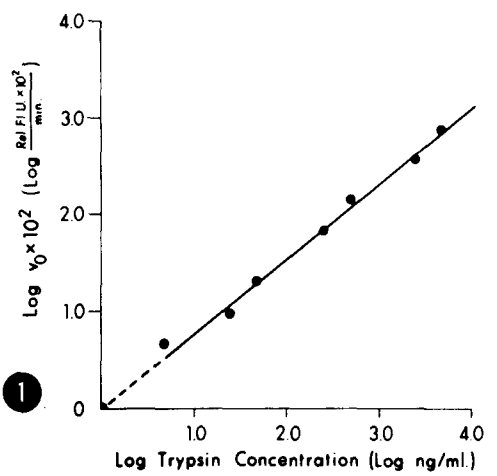
MATERIALS & METHODS

Materials: The following were the sources for the reagents used: Trypsin and α-chymotrypsin, both 2 X crystallized, salt free (Worthington Biochemical Corporation); 1-fluoro-2,4-dinitrobenzene (Eastman Organic Chemicals); n-acetylimidazole (Aldrich Chemical Company, Inc); purified thrombin (free of plasmin and clotting factors); protamine sulfate from herring (clupeine, essentially histone free); dinitrophenylalanine (Sigma Chemical Company). Fluorescamine was kindly supplied by Dr. Sidney Udenfriend of the Roche Institute, Nutley, N.J.

Preparation of Dinitrophenylprotamine Sulfate (DNPP): Protamine sulfate with a blocked amino terminal group was used in order to eliminate any fluorescence of the starting product. The DNPP was synthesized by adding 1 volume of 1-fluoro-2,4-dinitrobenzene in methyl cellusolve (0.1 M) to 5 volumes of a 1% solution of protamine sulfate in 0.01 M sodium tetraborate, pH 9.2. After an incubation at 37° C the resultant yellow precipitate was collected by centrifugation at 1000 x g for ten minutes, then washed twice with 20 ml of absolute ethanol and twice with 20 ml of acetone. The DNPP was dried at room temperature.

The DNPP gave an average molecular weight of 4400 as determined by optical density determination at 250 nm on a Gilford spectrophotometer. Dinitrophenylalanine was the standard. This material gave no fluorescence with fluorescamine, indicating complete blocking of the amino terminal groups. Amino acid analysis of protamine sulfate was done on a Jeolco Model JLC-5AH Automatic Amino Acid Analyzer.

Assay of Proteolytic Enzymes: The two main steps of this assay are: (1) Enzymatic hydrolysis of DNPP to liberate amino groups, and (2) Reaction of the free amines with fluorescamine to yield a fluorescent product.



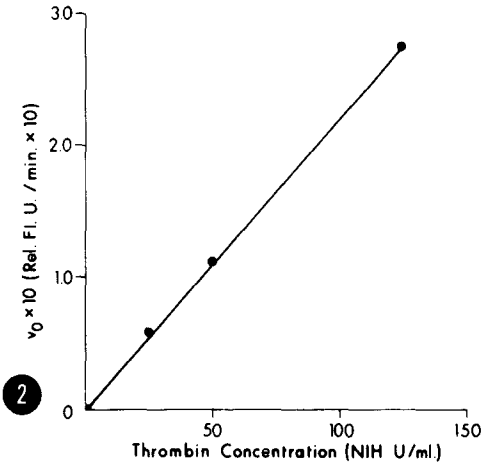


Figure 1: Initial Velocity vs. Concentration - Assay was performed as described in Methods. Trypsin concentrations ranged from 5 ng/ml - 5 µg/ml. Initial velocity values were based on fluorescence at 20 minutes and are expressed as the log of relative fluorescence units per minute X 10^2. Trypsin concentration is expressed as the log of micrograms per ml of incubation mixture X 10^3.

Figure 2: Thrombin: Initial Velocity vs. Concentration - Assay was performed as described in Methods except 1) DNPP - 5 ng/ml. 2) 0.01 ml aliquot + 0.4 ml buffer + 0.2 ml fluorescamine were used. Initial velocity is expressed as relative fluorescent units per minute X 10. Thrombin concentration as expressed as NIH units per ml of incubation mixture.

The incubation mixture (0.1 ml) consisted of equal volumes of enzyme solution in 10^{-3} M HCl and DNPP (10 mg/ml in 0.1 M sodium phosphate buffer, pH 7.5). Incubations were performed at 37.5° C. Aliquots (0.01 ml) were taken at twenty minutes and mixed with 0.6 ml of 0.1 M sodium phosphate, pH 7.0. While mixing vigorously at room temperature, 0.2 ml fluorescamine (1 mg per 10 ml analytical grade acetone dried over magnesium sulfate) was added.

Fluorescence was determined on a Perkin Elmer MPF-3 fluorometer standardized with a fluorescent standard (p-terphenyl gel, $\sim 3 \times 10^{-5}$ M) at an excitation wavelength of 390 nm, an emission wavelength of 470 nm, and a slit width such that a 1.35×10^{-6} M solution of quinine sulfate in 0.1 N sulfuric acid gave one relative fluorescence unit.

RESULTS

This method was capable of detecting small concentrations of arginine splitting proteases: trypsin, thrombin, and pepsin (1). Chymotrypsin, on the other hand, was totally inactive. This is to be expected as chymotrypsin preferentially hydrolyzes aromatic amino acid peptides (1).

Trypsin was detectable at concentrations as low as 5 ng/ml at 20 minutes. The rate of hydrolysis was proportional to enzyme concentration over a thousand-fold range (Figure 1). After twenty minutes the rate of hydrolysis decreased (data not shown). This assay, therefore, did not prove useful for incubation times longer than twenty minutes with trypsin.

Thrombin was capable of hydrolysing the DNPP at a concentration as low as 8.3 NIH units/ml at 40 minutes. The rate of hydrolysis was proportional to enzyme concentration over at least a five-fold range (Figure 2). After 40 minutes the rate of hydrolysis decreased (data not shown). Both thrombin and trypsin were inhibited by 4.5×10^{-3} M n-acetylimidazole (Table I).

Amino acid analysis of the protamine sulfate used in these studies is shown in Table II and compared to that previously reported (2).

DISCUSSION

Protamine, with its amino terminal group blocked with DNFB, offers an advantage as a substrate for trypsin-like enzymes. It did not yield any fluorescence with fluorescamine and was stable for months at room temperature.

TABLE I

EFFECT OF n-ACETYLIMIDAZOLE ON TRYPSIN AND THROMBIN

	Enzyme	n-acetylimidazole	Percent inhibition
Trypsin	5 μg/ml	4.5×10^{-3}M	94%
Thrombin	63 U/ml	4.5×10^{-3}M	83%
Thrombin	13 U/ml	4.5×10^{-3}M	83%

Enzymes were incubated with 4.5×10^{-3} M n-acetylimidazole (dissolved in benzene and evaporated to dryness) or with no inhibitor (controls) for 20 minutes. Aliquots of 0.01 ml were then assayed as described in Figure 2.

TABLE II

AMINO ACID COMPOSITION OF PROTAMINE SULFATE FROM HERRING

	Ando and Suzuki (2) Percent of Total	Present studies Percent of Total
ARG	67.0	65.8
ALA	7.7	7.2
SER	7.7	7.4
PRO	7.7	9.2
ILEU	1.1	1.4
THR	3.3	3.3
GLY	1.1	1.4
VAL	4.4	4.3

Hydrolysis was measured by determination of fluorescence without precipitation of the substrate in contrast to the more conventional protease assays using hemoglobin and casein (5, 6). This assay seems to offer an advantage over

sensitive synthetic substrates such as tosylarginine methyl ester (7), as it is more stable, exhibits no spontaneous hydrolysis, and measures hydrolysis of a peptide bond rather than an ester bond.

This assay demonstrates that thrombin is capable of splitting peptide bonds in addition to those in fibrinogen. Ando and Suzuki have reported (2) that there are no arginyl-glycine bonds in herring protamine, Clupea pallasii. Amino acid analysis results on the protamine that we have used are in close agreement with calculations done on Ando and Suzuki's reported structure. If the two are found to be identical, this would demonstrate that thrombin is capable of splitting peptide bonds other than the arginyl-glycine bonds hydrolyzed in fibrinogen (4).

N-acetylimidazole has been shown to inhibit the clotting activity of thrombin, but not its esterase activity (8). In our assay system, n-acetyl-imidazole inhibited proteolysis. This suggests that the clotting and proteolytic activities of thrombin are allied.

ACKNOWLEDGEMENTS

We thank Dr. Sidney Udenfriend for his kind gift of fluorescamine, and Dr. E.B. Ong for performing the amino acid analysis. This investigation was supported by USPHS NIH Grants AM 13532, ES 00606, and Center Grant ES 00260.

REFERENCES

1. White, A., Handler, P., and Smith, E.L, in Principles of Biochemistry, McGraw-Hill Book Co., New York, pp. 144, 726, & 734 (1968).

2. Ando, T. and Suzuki, K. Biochim. Biophys. Acta 140, 377-380 (1967).

3. Udenfriend, S., Stein, S., Bohlen, P., and Dairman, W. Science 178, 171-172 (1972).

4. Magnusson, S. in The Enzymes, Vol. III, Hydrolysis: Peptide Bonds (P.D. Boyer, ed), Academic Press, New York, pp. 295-296 (1971).

5. Anson, M.L., J. Gen. Physiol. 22, 79 (1938).

6. Moore, G.L., Kocholaty, W.F., Cooper, D.A., Gray, J.L., and Robinson, S.L. Biochim. Biophys. Acta 212, 126-133 (1970).

7. Roffman, S., Sanocka, U. and Troll, W. Anal. Biochem. 36, 11-17 (1970).

8. Lundblad, R.L., Harrison, J.H., and Mann, K.G. Biochem. 12, 409-413 (1973).