SPECTROFLUOROMETRIC ASSAY FOR ELASTOLYTIC ENZYMES[1]

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Received June 9, 1970

SUMMARY

Soluble products of elastin proteolysis exhibit fluorescence at 390 nm (λ excitation 320 nm). A specific and sensitive assay has been developed using the intensity of this fluorescence as a measure of elastolysis.

Enzymes which degrade the insoluble structural protein, elastin, are elaborated by the mammalian pancreas and by bacteria and molds (1). Pancreatic elastase was first described twenty years ago by Balo and Banga (2), but studies of the characteristic physical and chemical properties of this and other elastases have been hampered by the lack of a specific, sensitive, and rapid assay system utilizing unmodified elastin as a substrate. Procedures previously used to measure elastolytic activity include measurements of the solubilization of elastin by gravimetric methods (3), by the biuret reaction (4), and by production of a clear zone on elastin-agar (5). These assay methods are relatively lengthy, insensitive, and require large amounts of enzyme. Elastins modified by binding of dyes, such as Congo red (6), orcein (7), fluorescein and Remazolbrilliant Blue (8), also have been used as elastase substrates. However, the site and mechanism of dye binding to elastin is not

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[1]We gratefully acknowledge the support of this work in part by U.S. Public Health Service Grant AM-07300.

[2]RSQ is the holder of a Graduate Fellowship for Women from the Danforth Foundation.

understood, and alterations in enzyme binding and mode of action may result from the presence of a foreign chromophoric moiety.

Recently, more sensitive elastase assays using synthetic ester substrates have been developed (9,10,11), but one cannot rely solely on demonstrated activity against these substrates to assign elastolytic activity for, by definition, an elastase must degrade elastin. Furthermore, the physiological relevance of effects observed with synthetic substrates must be confirmed by use of an enzyme's natural substrate. We have developed an assay using unmodified elastin as the substrate, which is not subject to the limitations of other methods. This specific and sensitive assay is based upon measurement of the intensity of the characteristic fluorescence of the soluble peptide fragments of elastin produced by elastolysis.

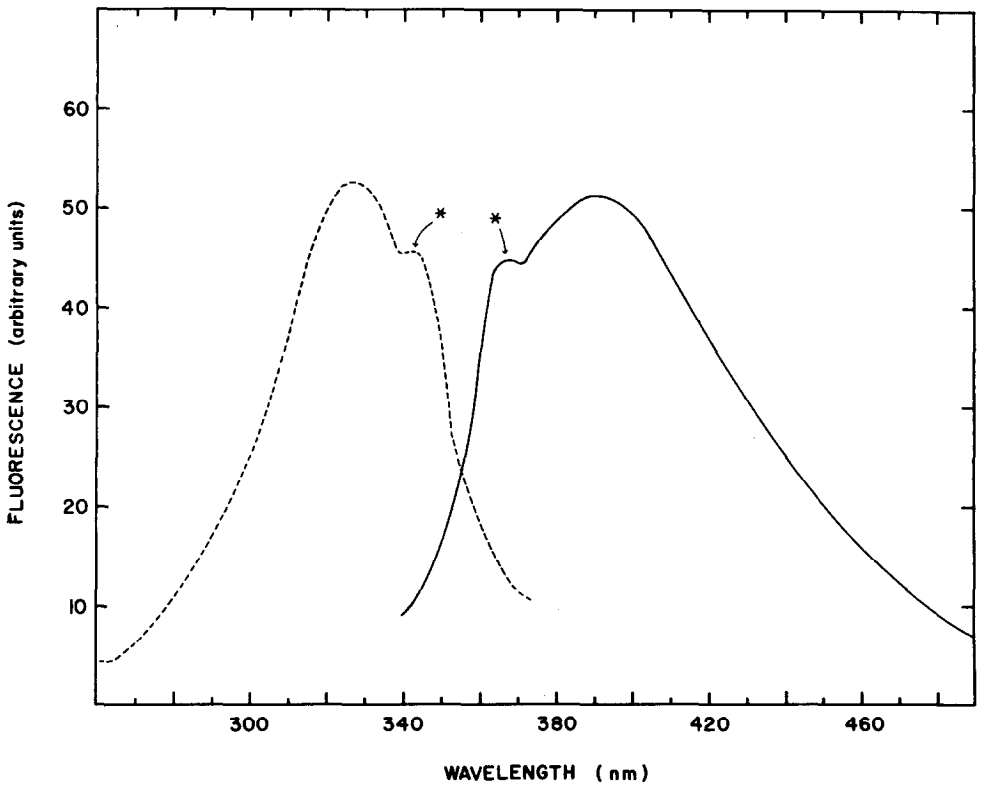

Figure 1: Excitation and emission spectra of soluble products of elastase-treated elastin. ---- indicates excitation spectrum (emission λ 390 nm). ——— indicates emission spectrum (excitation λ 320 nm). * indicates H_2O Raman scatter peak.

EXPERIMENTAL

Elastin (bovine ligamentum nuchae, Lot EL 9CA), porcine elastase (Lot ESFF 9DA), trypsin (Lot TRSF 6FA), α-chymotrypsin (Lot CDI 6JF), and papain (Lot PAP 7DA) were purchased from the Worthington Biochemical Corporation. Pronase (Lot 502117) was obtained from Calbiochem. Subtilisin (Lot P52B-082) was purchased from Sigma Chemical Company. All buffer reagents were of analytical grade.

Fluorescence measurements were made with an Hitachi-Perkin Elmer Model MPF-2A Spectrofluorimeter utilizing the high sensitivity sample cell holder and ratio recording mode. The spectral maxima are uncorrected for the response of the type R 106 photomultiplier tube.

RESULTS AND DISCUSSION

Fluorescence Spectra: The fluorescence activation and emission spectra of the soluble products of elastolysis of bovine ligamentum nuchae elastin by elastase are presented in Figure 1. The characteristic and intense fluorescence emission peak at 390 nm (λ excitation 320 nm) increases rapidly with time of incubation of elastin with pancreatic elastase (Figure 2).*

Assay Method: Between ten to thirty mg of elastin are weighed, and two ml of 0.05M Tris buffer (pH 8.8) are added to the protein in a test tube. A measured volume of the enzyme solution to be assayed is added to the tube, and the resulting mixture is incubated for 30 minutes at 37°C with shaking. Then two to four ml of 1M NaCl in 0.2M acetate buffer (pH 5.6) are added to stop the reaction. The assay mixture is spun down in a clinical centrifuge (at ~3300 rpm) until the solution appears clear (5 to 10 minutes), and the resultant fluorescence of the supernatant is measured at 390 nm (λ excitation 320 nm). There is a linear relationship between the fluorescence observed (under these conditions) and enzyme concentration (Figure 3).

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*Buffer incubated with elastin shows a small amount of background fluorescence which should be subtracted from the measured fluorescence. The data shown in Figure 2 are not corrected for this background fluorescence.

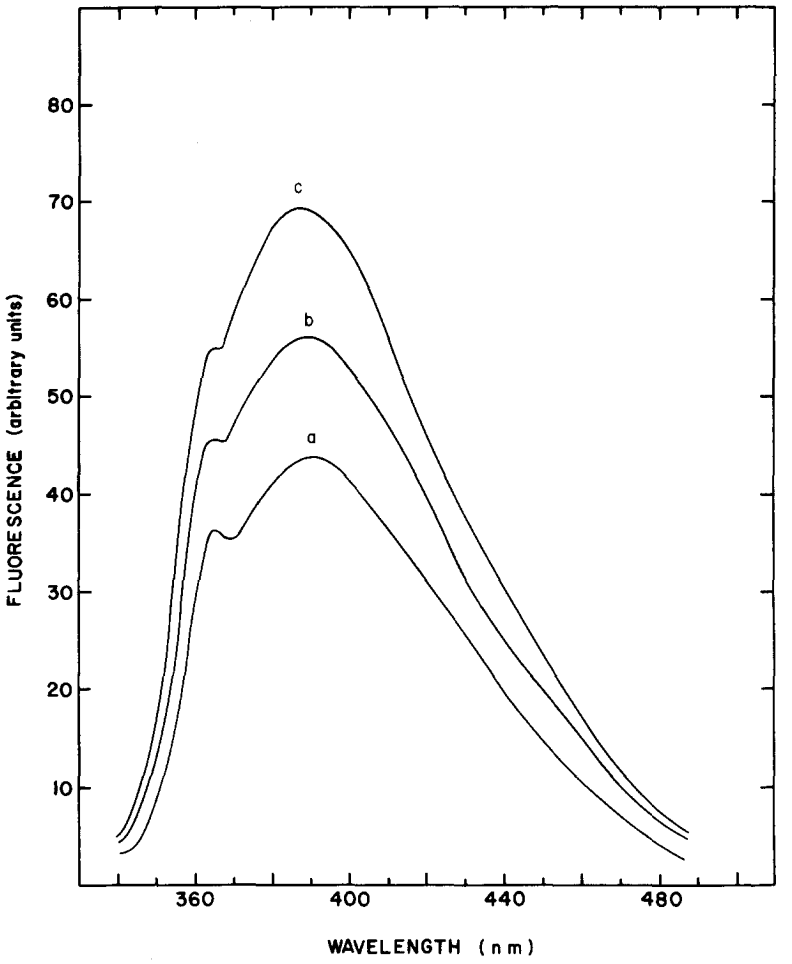

Figure 2: Recorder traces of fluorescence emission spectra of soluble products of elastase-treated elastin. Elastin (15 mg/ml) incubated with elastase (12.5 μg/ml) at 37°C for: a) 5 minutes b) 10 minutes c) 15 minutes

Discussion: This assay method is sensitive. As little as one microgram of pancreatic elastase produces measurable fluorescence after thirty minutes incubation at 37°C. It is possible that even smaller amounts of this enzyme can be detected by increasing the incubation time.

The assay has been used successfully with crude pancreatic tissue homogenates and should, in principle, be applicable to the determination of elastolytic activity in other tissue homogenates and enzyme mixtures. The presence of proteins containing tyrosine (λ excitation 270±20 nm, emission

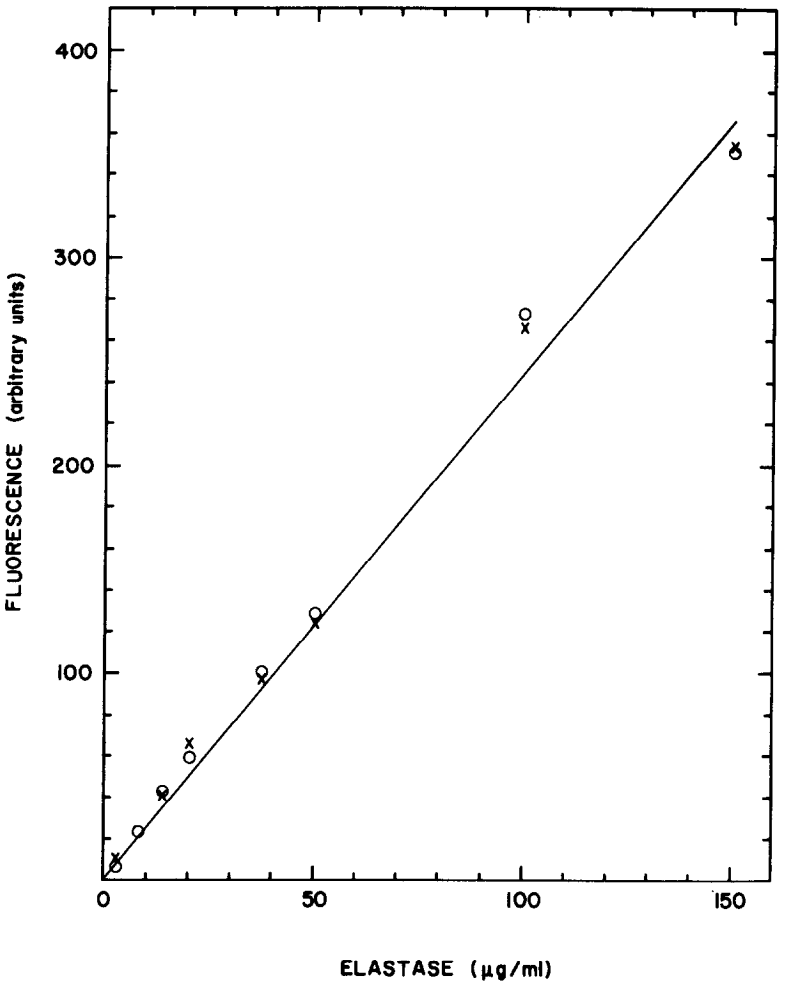

Figure 3: Variation of fluorescence intensity (390 nm) with elastase concentration. Two ml of a mixture of 0.05M Tris buffer, pH 8.8, and bovine ligamentum nuchae elastin (15 mg/ml) incubated with indicated concentrations of elastase at 37°C for 30 minutes. (O) and (X) represent average results from two different series of determinations.

~303 nm) and tryptophan (λ excitation 270±30 nm, emission 320 to 355 nm) will not interfere with fluorescence emission intensity measurements at 390 nm (λ excitation 320 nm). To remove extraneous protein from such mixtures, we have found it is best to use heat precipitation (exposure to 100°C for 3 to 5 minutes); treatment with trichoroacetic acid (5%) cannot be used without concomitant precipitation of peptide products contributing to the fluorescence. One must be aware that the presence in the assay solution of substances which

quench fluorescence could interfere with this assay, though we have not encountered this situation.

This assay has been used to investigate elastolytic activity of a number of enzymes which have been reported to digest elastin (5). The non-specific bacterial proteases, pronase (0.4 mg/ml) and subtilisin (0.7 mg/ml), show activity against elastin after one hour incubation; however, α-chymotrypsin (0.3 mg/ml), trypsin (0.9 mg/ml), and papain (0.5 mg/ml) produce no measurable increase in fluorescence during this time. After prolonged incubation (24 hours) at 37°C, the latter three enzymes show small, but measurable, activity which could possibly be due to contamination with a minute amount of an elastolytic enzyme.

This assay is presently being used to investigate the properties of pancreatic elastase. Progress has been made in the identification of the elastin moieties which contribute to the observed fluorescence, and these results will be reported in due course.

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