[Chem. Pharm. Bull. 30(2) 644—651 (1982)]

Basic Study on the Fluorometric Determination of Elastase Activity

Yoshiyuki Seyama, Eiji Usami and Saburo Yamashita*

Department of Clinical Chemistry, Hoshi College of Pharmacy, 2-4-41 Ebara, Shinagawa-ku, Tokyo, 142, Japan

(Received July 10, 1981)

An assay method for elastase activity was established using a natural substrate, elastin, because synthetic low molecular weight substrates may not reflect elastase activity accurately.

A fluorometric assay method with fluorescamine was used, after a deproteinizing process with trichloroacetic acid. Neutralization of 5% trichloroacetic acid was done with 0.2 N NaOH, and the fluorescence of the products formed from elastin by elastase with fluorescamine was determined in dioxane–phosphate buffer after the enzymatic reaction had been carried out in H_3BO_4–NaOH buffer, pH 8.8, with 10 mg elastin/1.1 ml of assay mixture.

As little as 1.56×10^{-5} mg of elastase can be determined by the present method, and there was a good correlation between the results of a conventional method using succinyl alanylalanylalanyl-*p*-nitroanilide and the present method.

Keywords——elastin; elastase; fluorescamine; synth. substrate; natural substrate

Since succinyl alanylalanylalanyl-*p*-nitroanilide (suc(Ala)$_3$*p*NA)[1] was introduced as a specific synthetic substrate of elastase, the presence and isolation of elastase activity in various biological materials have been reported. However, it has been proved that there are some enzymes which hydrolyze only the synthetic substrate but not elastin.[2,3] Therefore, activity to hydrolyze the synthetic substrate is inadequate as a criterion of true elastase activity. In order to determine the true physiological properties of elastase, the use of the natural polymeric substrate, elastin, is essential.

This paper deals with a basic study on the method of determining the activity of elastase using elastin as the substrate.

Largman and co-workers[4] reported a fluorometric determination method for elastase II activity after separating and purifying elastase I and elastase II. Some improvements to their method seemed desirable, so we carried out some basic examinations of the procedure.

Materials and Methods

Substrate——Elastin (from bovine neck ligament) was obtained from Sigma Chem. Co., St. Louis, Mo., U.S.A., and prepared as 200—400 mesh fine powder by means of an Electromagnetic Laboratory Micro-Pulverizer (Fritsch, Germany). Suc(Ala)$_3$*p*NA was obtained from the Peptide Inst., Protein Research Foundation Inc., Minoh, Osaka.

Enzymes——Elastase used in the present experiment was obtained from Millipore Corp., Freehold, N.J., U.S.A., and the activity was labelled as 9 U/mg (one unit of the activity is defined as that amount of enzyme converting 1 μmol of *N*-acetyl-Ala$_3$NA per min at 25°C as described by Feinstein *et al.*[5]

Trypsin——TPCK was obtained from Millipore Corp., and labelled as 238 U/mg, α-chymotrypsin was from Millipore Corp., labelled as 49.2 U/mg. Collagenase was from Millipore Corp., prepared from *Clostridium histolyticum* and labelled as 341 U/mg.

Inhibitors——Diisopropyl fluorophosphate (DFP) was obtained from Sigma Chem. Co. Elastatinal was obtained from the Peptide Inst., Protein Res. Foundation. Human α_1-antitrypsin was obtained from Millipore Corp., and labelled as "1 mg inhibits 146 mg trypsin." Trypsin inhibitor, from chicken egg white Type II-O, was obtained from Sigma Chem. Co., and labelled as "1 mg will inhibit approx. 1 mg trypsin with activity approx. 10000 BAEE/mg protein and 1 mg will inhibit approx. 0.3 mg thymus trypsin with activity approx. 40 BTEE/mg protein." Lignin sulfonic acid sodium salt and NaCl were obtained from Tokyo Kasei, Tokyo.

Protein——Bovine serum albumin (BSA) Fraction V was obtained from Sigma Chem. Co.

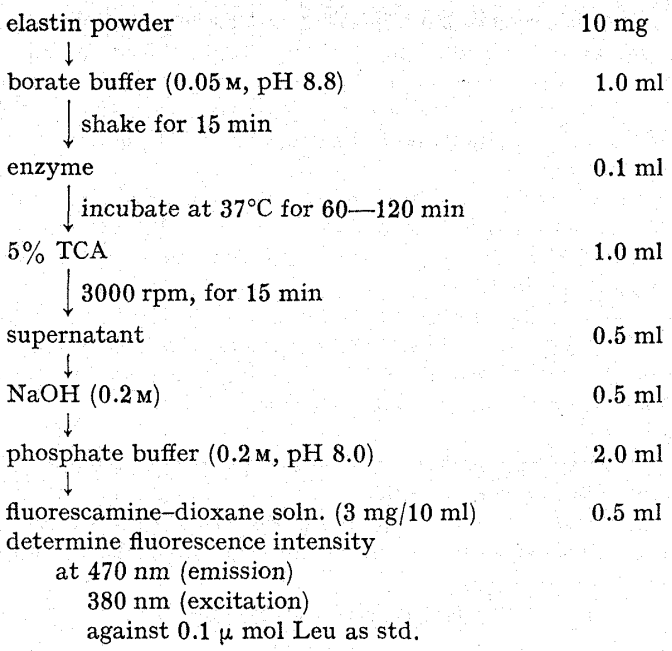

Fig. 1. Outline of the Assay Procedure for Activity of Elastase and Other Elastolytic Enzymes

Fluorogenic Reagent——The reagent used was fluorescamine (4-phenylspiro-[furan-2(3*H*),3′(1′*H*)-(2′-benzofuran)-3,1′-dione) (Fluram, Basel, Switzerland).

Assay Method for Elastolytic Activity——General enzymatic activities were determined according to the method shown in Fig. 1.

Examination of the Experimental Conditions : Fluorogenic Conditions——Fluorescamine, 3.0 mg, was dissolved in 10 ml of acetone or dioxane. A portion, 0.5 ml, of the solution was mixed with 3.0 ml of 0.2 M phosphate buffer, pH 8.0, or of 0.2 M H_3PO_4–NaOH buffer, pH 8.0, and the reagent blank fluorescence intensity was determined at 450 nm emission wavelength (excited at 380 nm). pH's of the buffer solutions were changed to 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0 and aliquots of the hydrolyzate of elastin with elastase were mixed with buffers of various pH's. Fluorescamine–dioxane solution, 3 mg/10 ml, was added to establish the fluorogenic conditions.

Determination of the Concentration of Trichloroacetic Acid (TCA)——Mixtures of equal amounts of 0.05 M borate buffer, pH 8.8, and various concentrations, 5, 10, 15 and 20%, of TCA were prepared. To the mixture, various concentrations, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 M, of NaOH solution in 0.5 ml were added. Then, 2.0 ml of 0.2 M phosphate buffer, pH 8.0, an aliquot of elastin hydrolyzate with elastase, and 0.5 ml of fluorescamine–dioxane solution were mixed. The amount of TCA required to neutralize NaOH and to provide optimum fluorogenic effect was determined by this method.

Human serum, 100 μl, was dissolved in 1.0 ml of 0.05 M borate buffer, pH 8.8, and various concentrations of TCA, 5, 10, 15 and 20%, (1.0 ml), were added. After centrifugation, an aliquot was neutralized with the above-mentioned NaOH, and the fluorescence intensity was determined by the method shown in Fig. 1. Taking the fluorescent intensity as 100% (control) when no TCA was added, the fluorescence intensities obtained with TCA were converted to the relative intensities (per cent of the control value) and thus the efficiency of deproteinization by various concentrations of TCA was obtained.

An aliquot of the hydrolyzate of elastin with elastase (0.2 μg, and 1.0 μg of elastase) was mixed with 1.0 ml of 0.05 M borate buffer, pH 8.8, and then various amounts of TCA (5, 10, 15, and 20%) were added to the mixture. The hydrolyzed soluble products were treated by the method shown in Fig. 1, and the fluorescence intensity was determined. The optimum concentration of the hydrolyzate was obtained in conjunction with the optimum amount of TCA to remove protein and to produce the maximum fluorescence intensity.

To check the interference by protein, bovine serum albumin (BSA) (100—1000 μg) was added to a mixture of elastin hydrolyzate (0.1 μg, 0.2 μg, 1.0 μg) and 0.05 M borate buffer, pH 8.8, and the fluorescence intensity was determined after removing protein with 5% TCA. The results are expressed as per cent fluorescence intensity relative to the control (containing no BSA).

Determination of the Incubation Conditions——Elastin hydrolyzing activity with elastase (0.25 μg, and 0.5 μg) was compared in various buffer solutions at pH 8.8 (0.5 M H_3BO_4–NaOH, 0.05 M $NaHCO_3$–HCl, and 0.05 M Na borate–HCl), and the activity was also compared in 0.05 M H_3BO_4–NaOH and in 0.05 M triethanolamine hydrochloride containing 0.025% Triton X-100 (Largman's method). The pH's of 0.05 M H_3BO_4–

NaOH were adjusted to 7.5, 8.0, 8.5, 8.8, 9.0, and 10.0 and the elastin hydrolyzing activity with elastase was compared. Various amounts of the substrate, elastin (2.5, 5, 10, 20, 30, and 40 mg) were suspended in 1.0 ml of 0.05 M H_3BO_4–NaOH buffer, pH 8.8, and elastase activity with 0.25 μg and 1.0 μg was determined. The ratio of the substrate to the enzyme was also changed; that is, 0.125 μg, 0.5 μg, or 1.0 μg of elastase was added to 10 mg of elastin and the mixture was incubated for 30, 60, 90, 120, and 180 min. The time courses of elastase activity were determined.

Comparison of Elastin Hydrolyzing Activities of Elastase and Other Proteolytic Enzymes——Elastase hydrolyzing activity of elastase was determined with 0.0625, 0.125, 0.25, 0.5, or 1.0 μg of elastase according to the method described in Fig. 1, and those of trypsin, α-chymotrypsin and collagenase with 0.5, 1, 2 or 10 μg of the enzyme. Incubation was performed for 2 h.

Inhibitors of Elastase——Various inhibitors of elastase activity, such as lignin, elastatinal, α_1-antitrypsin, trypsin inhibitor, NaCl, or DFP, were mixed with the incubation mixture for elastase assay: 10 mg of elastin in 0.05 M borate buffer, pH 8.8, as a suspension, with 0.5 μg of elastase, and the mixture was preincubated in a cold water bath for 30 min, and then in a water bath at 37°C for 2 h. The hydrolyzed products were determined fluorometrically.

Correlation of Elastin Hydrolyzing Activity and suc$(Ala)_3$*p*NA Hydrolyzing Activity——The activities were determined, and the correlation was obtained.

Fluorometric Data——Fluorometric data were expressed in one of two ways: 1) intensity of fluorescein, and 2) relative fluorescence intensity with respect to the intensity of the standard, Leu (μmol).

Results and Discussion

Determinations of the activity of elastase have been reported from various laboratories, and the methods may be classified into the following two kinds of procedure: (I) pretreatment of the substrate elastin with dyestuff,[6] with fluorogenic compounds,[7,8] or with radioisotopes,[9–11] and (II) detection of hydrolyzed products of the substrate by spectrophotometric method (UV),[12] fluorescence,[13] or by ninhydrin treatment.[14,15]

Elastin is a water-insoluble substrate of elastase, and water-soluble peptides are released by the action of the enzyme. Fluorescamine reacts with the split peptides to give fluorescent material.

(1) Fluorogenic Conditions

Fluorescamine is dissolved in an organic solvent, and reacts with peptides split from the substrate, elastin, by the action of elastase in an alkaline medium. Various combinations of fluorescamine solution, solvents, and buffer solutions, pH 8.0, were examined to obtain the optimum fluorogenic conditions and to minimize the reagent blank. It was found that the best solvent-buffer combination was dioxane–phosphate buffer, followed by acetone–phosphate buffer (Largman method), dioxane–borate buffer, acetone–borate buffer in that order (in terms of reagent blank value). The CV (coefficient of variation) was the least for dioxane–phosphate buffer, and became larger in the order of dioxane–borate buffer, acetone–phosphate buffer, and acetone–borate buffer (Fig. 2); that is, the experimental variance of the blank value is least with dioxane–phosphate buffer, so that this combination of fluorescamine solvent–buffer solution was used throughout the present experiments. The results obtained with this fluorescamine solvent–buffer solution system in the present study were found to be better than those obtained by the Largman method.

Then, the fluorogenic efficiency of fluorescamine with the soluble reaction products of elastin split by the action of elastase was examined. The fluorescence intensity with fluorescamine and the enzymatic reaction products was maximum at pH 7.5—8.0 in phosphate or borate buffer; the fluorescence intensity was stable in phosphate buffer over a wide range of pH, 7.5 to 9.0, whereas the fluorescence intensity was only stable in a narrower pH range, pH 7.5 to 8.0, in borate buffer (Fig. 3). Therefore, fluorescamine was dissolved in dioxane and then reacted with the enzymatic reaction products in 0.2 M phosphate buffer, pH 8.0.

(2) Concentration of TCA for Precipitating Protein

When serum elastase is used, protein interferes with fluorogenesis. TCA is used for deproteinization in the present study. TCA concentration must be restricted to a level just

sufficient to precipitate the serum protein, and not the reaction products. Since the fluorogenic reaction is performed at pH 8.0, the amount of sodium hydroxide required for the neutralization of TCA was examined in the presence of the elastin hydrolysate with elastase. The fluorescence intensity was greatest when 5% TCA was neutralized with 0.2 N NaOH. When 2.5% TCA was used, 0.05 N NaOH was suitable (0.6 N NaOH for 15% TCA, and 0.7 N NaOH

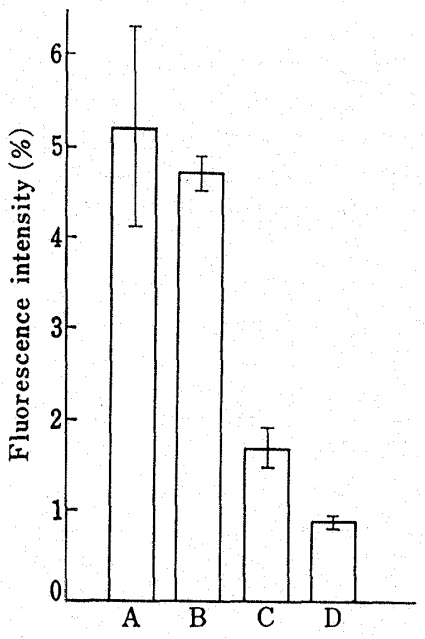

Fig. 2. Comparison of the Fluorescence Blank Values in Various Solvent and Buffer Solutions ($n=5$) (Mean ± S.D.)

A: acetone–borate (CV% = 42.8).
B: dioxane–borate (CV% = 9.75).
C: acetone–phosphate (CV% = 40.4).
D: dioxane–phosphate (CV% = 4.8).

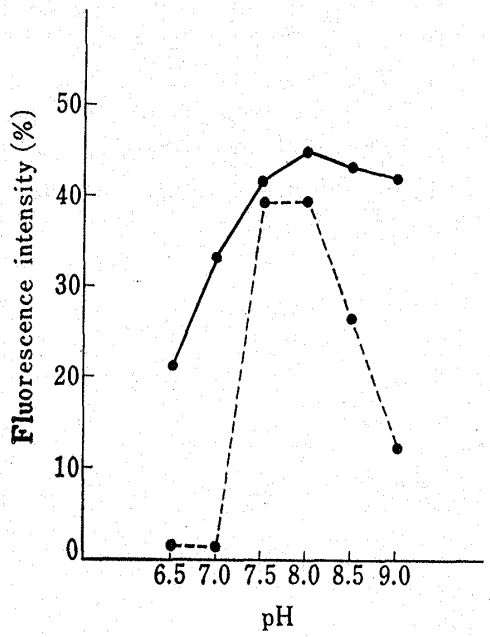

Fig. 3. Effect of pH of the Fluorogenic Procedure on Elastin Hydrolysate split by Porcine Elastase

•——•: phosphate buffer.
•- - -•: borate buffer.

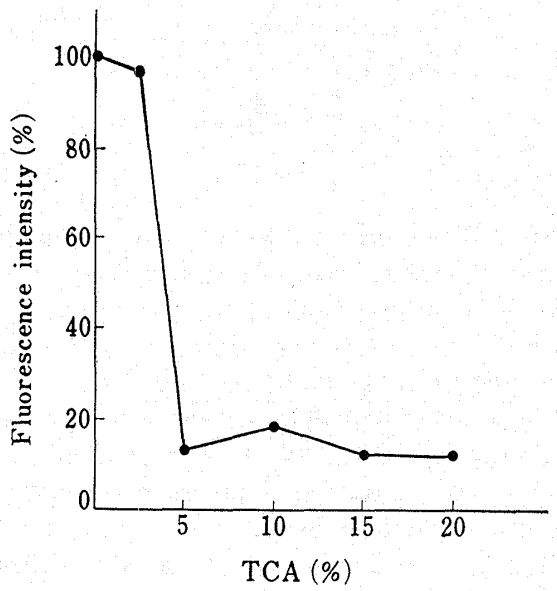

Fig. 4. The Optimum Concentration of TCA for Precipitation of Serum Protein ($n=2$)

100% is the value obtained without TCA.

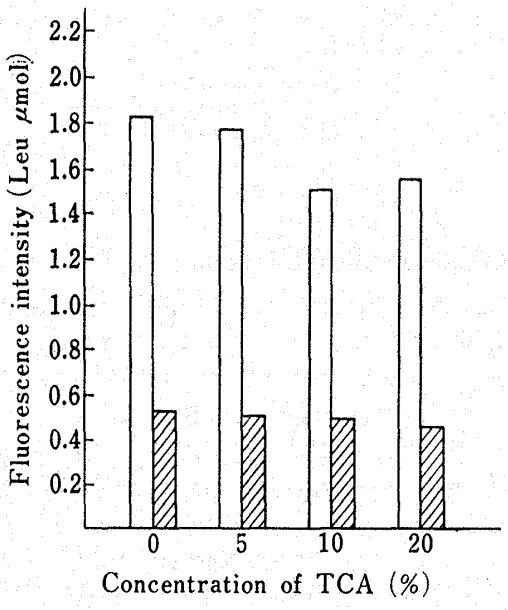

Fig. 5. Effect of TCA Concentration on the Precipitation of Elastin Hydrolysate by Elastase

The time of incubation was 2 h.
□: 1 μg elastase.
▨: 0.2 μg elastase.

for 20% TCA). The deproteinizing effect of TCA in the presence of 100 μl of serum was examined, as shown in Fig. 4. The effect was almost the same at TCA concentrations above 5%. The precipitating effect of 5% TCA on the elastin hydrolysate with elastase was negligible; that is, elastin hydrolysate was not precipitated, and the fluorescence intensity of the hydrolysate with and without 5% TCA was almost the same; however, 10 or 20% TCA gave lower intensity (Fig. 5). Therefore, it may be concluded that 5% TCA will not precipitate the elastin hydrolysate but will precipitate the serum protein.

The possibility of coprecipitation of protein and elastin hydrolysate was also examined. Elastin hydrolysate obtained with 0.5 μg, 0.2 μg or 0.1 μg of elastase was not precipitated with 5% TCA in the presence of BSA (bovine serum albumin, 100—1000 μg), and only BSA was removed by TCA precipitation (Fig. 6). Therefore, the method presented here is not affected by the presence of serum protein, and appears to be superior to the Largman method, in which no deproteinizing procedure is used.

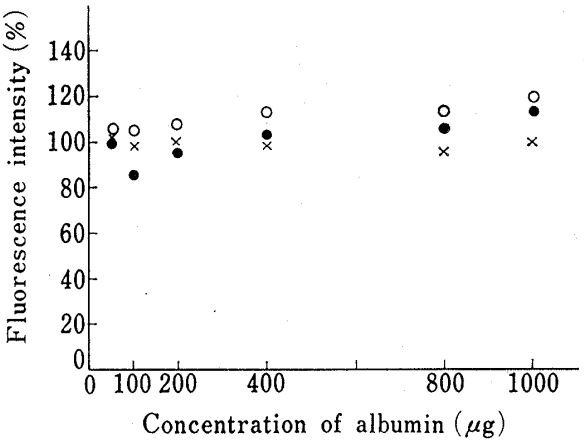

Fig. 6. Effect of externally Added Albumin on Elastase Assay by the Method presented in This Paper

×: 0.1 μg elastase.
○: 0.2 μg elastase.
●: 1.0 μg elastase.

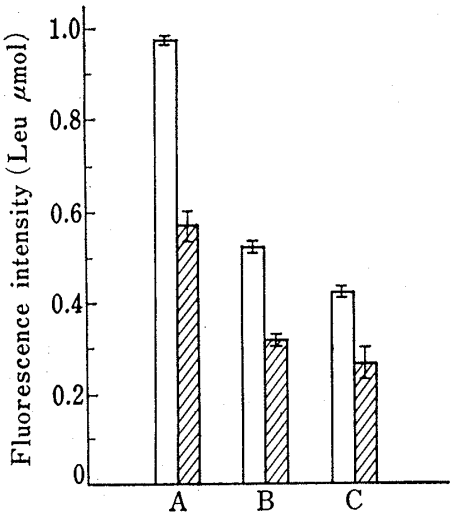

Fig. 7. Effect of the Buffer Combination on the Activity of Elastase ($n=6$)

Time of incubation was 2 h.
Buffer A: H_3BO_4–NaOH.
Buffer B: Na_2CO_3–HCl.
Buffer C: $Na_2B_4O_7$–HCl.
Enzyme □: 0.5 μg elastase.
Enzyme ▨: 0.25 μg elastase.

(3) Conditions of Incubation

Takahashi *et al.*[16] reported that the elastase activity was different for a synthetic substrate, Ala_3OMe, depending on the buffer used for the assay. Katayama[17] also reported such a difference in elastase activity for suc$(Ala)_3$*p*NA in various concentrations of Tris-buffer. Elastase activity was therefore examined at the same pH but in buffers with different constituents, such as H_3BO_4–NaOH, $NaHCO_3$–HCl, Na borate–HCl, *etc.* As shown in Fig. 7, the H_3BO_4–NaOH buffer was superior to other buffer system in terms of elastin hydrolyzing activity when 0.25 μg and 0.5 μg of elastase were used. It was found that the elastase activity with H_3BO_4–NaOH buffer (activity defined as 100%) used in the present study was higher than that in the buffer used by Largman (activity 80.7% of that in the present method).

The optimum pH of elastase was reported to be 8.8, and it was identified in the present study by using borate buffer and carbonate buffer. The optimum amount of the substrate was examined using 0.125 μg or 1.0 μg elastase, and it was found that substrate saturation was obtained with 5 mg of elastin for 0.125 μg elastase and with 10 mg of elastin for 1.0 μg of elastase: 10 mg of the substrate was used in the following assay (Fig. 8).

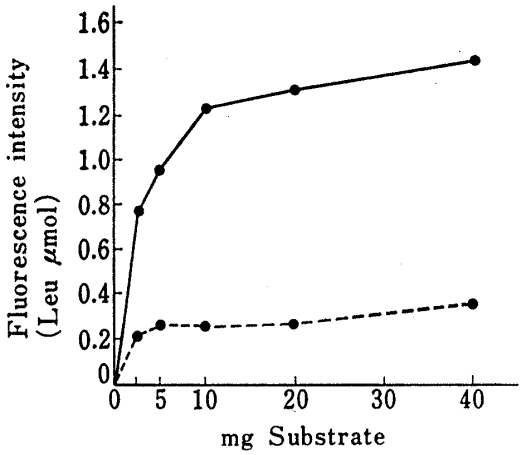

Fig. 8. Relationship between the Amount of Elastin and the Rate of Elastin Hydrolysis ($n=4$)

Incubation was performed for 1 h.
—●—: 1.0 μg elastase.
--●--: 0.125 μg elastase.

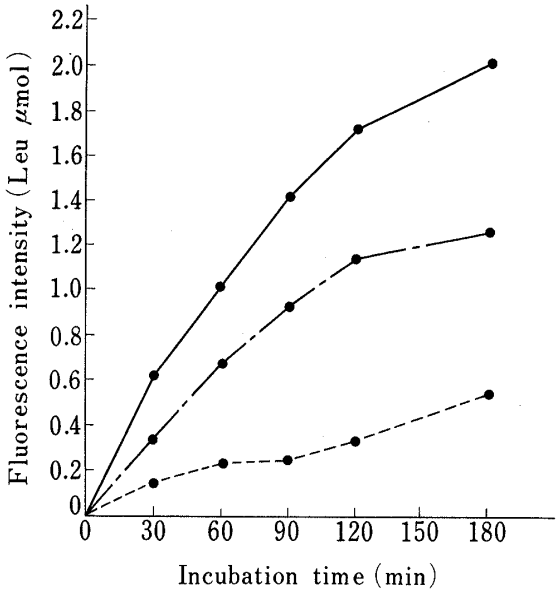

Fig. 9. Elastolytic Activity and Time of Reaction ($n=2$)

●——●: 1.0 μg elastase.
●–·–●: 0.5 μg elastase.
●-----●: 0.125 μg elastase.

Using these established conditions, elastin hydrolyzing activity and incubation time were examined with 0.125 μg, 0.5 μg and 1.0 μg elastase incubated for 30, 60, 90, 120 and 180 min at 37°C. The elastin hydrolyzing activity was linear up to 120 min of incubation. Therefore, the time of incubation was set at 120 min, or less (Fig. 9).

Using 10 mg of elastin, the effect of amount of elastase was examined. The amount of elastase used was 1 μg, 0.5 μg, 0.25 μg, 0.125 μg, or 0.0625 μg. The enzymatic reaction was performed for 120 min and it was found that the amount of enzyme and the fluorescence intensity obtained were linearly related.

The minimum detectable amount of the split substrate was 1.56×10^{-5} mg, which represents a sensitivity comparable to that obtained with radioisotope labelling. Elastolytic activities of trypsin, α-chymotrypsin, and collagenase were compared. As shown in Fig. 10, the elastolytic activity of collagenase was the lowest (negligible), whereas trypsin and α-chymotrypsin exerted elastolytic activity, although the activity was lower than that of elastase. Further purification of the substrate, elastin, will be necessary to clarify the substrate specificity of these enzymes.

Inhibitors of elastase activity have been reported, so that the inhibitory ratios of the inhibitors were examined. Inhibitory effects on elastase of DFP, NaCl, elastatinal, trypsin inhibitor, α_1-antitrypsin, and lignin were confirmed (Table I).

It was clarified that the results of assay of elastase activity using a natural substrate, elastin, and a synthetic substrate, suc$(Ala)_3$*p*NA were positively and significantly correlated (Fig. 11). Reproducibility (precision) of the present method was expressed as the coefficient of variation (CV), and it was found to be 5.4% ($n=10$), (1.48±0.08 Leu, when 0.5 μg elastase was used).

The assay method presented here provides a highly sensitive procedure for the determination of elastase activity (detection limits 1.56×10^{-5} mg of elastase) towards a natural substrate, elastin. Synthetic substrates may not give reliable activity values of elastase, but the present method should reflect *in situ* elastase activity.

It has been suggested that other proteases than elastase might hydrolyze the natural substrate, elastin. This may not be significant when pure elastase is used as in the present

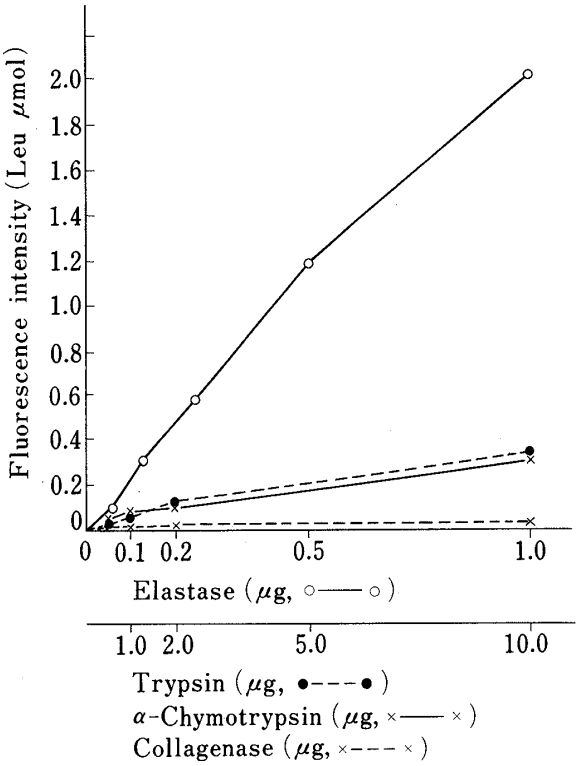

Fig. 10. Substrate Specificity of Elastase on Elastin ($n=2$)

Elastolytic activities of other proteolytic enzymes are negligible.
The time of reaction was 2 h.

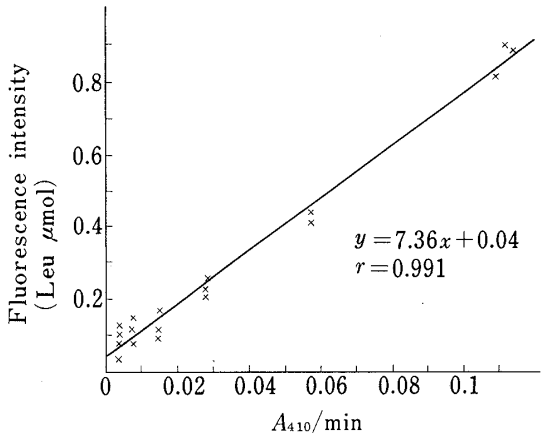

Fig. 11. Correlation of the Elastase Activities towards suc(Ala)$_3$$p$NA and Elastin

Ordinate: Elastin was used as the substrate (present method).
Abscissa: suc(Ala)$_3$$p$NA was used as the substrate.

Table I. Effects of Inhibitors on the Elastolytic Activity of Elastase

Inhibitor	Final concn.	Inhibition (%)
DFP	10^{-7} M	7.9
	10^{-5} M	88.3
	10^{-3} M	94.8
NaCl	6×10^{-2} M	50.0
	1.15×10^{-1} M	58.8
	5.0×10^{-1} M	85.0
Elastatinal	1 μg/1.1 ml	13.3
	10 μg/1.1 ml	48.3
	100 μg/1.1 ml	68.4
α_1-Antitrypsin	1 μg/1.1 ml	4.4
	10 μg/1.1 ml	29.0
	100 μg/1.1 ml	45.6
Trypsin inhibitor	1 μg/1.1 ml	11.5
	10 μg/1.1 ml	43.9
	100 μg/1.1 ml	57.9
Lignin sulfonic acid	1 μg/1.1 ml	7.4
	10 μg/1.1 ml	7.4
	100 μg/1.1 ml	17

method, but when the elastase activity in a biological specimen is determined, the presence of proteases other than elastase may give erroneous results. In such cases, a protease inhibitor(s) which will not alter the elastase activity should be added to the assay mixture.

Application of the present assay method for elastase activity to biological materials is now being undertaken and the results will be reported shortly.

References and Notes

1) J. Bieth, B. Spiess, and C.G. Wermuth, *Biochem. Med.*, **11**, 350 (1974).
2) A. Ito, J. Kobayashi, Y. Mori, and S. Hirokawa, *Biochem. Med.*, **23**, 195 (1980).
3) J. Saklatvala, *J. Clin. Invest.*, **59**, 794 (1977).
4) C. Largman, J.W. Brodrick, and M.C. Geokas, *Biochemistry*, **15**, 2491 (1976).
5) G. Feinstein, A. Kupfer, and M. Sokolovsky, *Biochim. Biophys. Res. Comm.*, **50**, 1020 (1973).
6) D.A. Hall, *Biochem. J.*, **59**, 459 (1955).
7) H. Rinderknacht, M.C. Geokas, P.S. Silverman, Y. Lillard, and B.J. Haverback, *Clin. Chim. Acta*, **19**, 327 (1968).
8) P.F. Huebner, *Anal. Biochem.*, **74**, 419 (1976).
9) S. Takahashi, S. Seifter, and F.C. Tang, *Biochim. Biophys. Res. Comm.*, **39**, 1058 (1970).
10) S.Y. Yu and A. Yoshida, *Biochem. Med.*, **21**, 108 (1979).
11) W. Bieger and G. Scheele, *Anal. Biochem.*, **104**, 239 (1980).
12) W. Ardelt and S. Ksiezng, *Anal. Biochem.*, **34**, 180 (1970).
13) R.S. Quinn and K.R. Blout, *Biochim. Biophys. Res. Comm.*, **40**, 328 (1970).
14) L. Fracois, C.P. Craig, and S. Tauber, *J. Biochem.*, **236**, 86 (1961).
15) Y. Misawa, K. Satake, and K. Okuda, *Rinsho Kagaku*, **5**, 176 (1977).
16) K. Takahashi, Y. Seyama, and S. Yamashita, *Chem. Pharm. Bull.*, **28**, 23 (1980).
17) K. Katayama, *Igaku no Ayumi*, **99**, 613 (1976).