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Simultaneous Determination of Esterase and Peptidase Activities of Elastase

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1. Synthetic substrates conventionally used can give either the esterase or peptidase activity of elastase separately, but simultaneous determination of both activities using one substrate has not previously been attempted.

2. In this work, the esterase and peptidase activities of elastase were determined simultaneously.

Esterase activity can be expressed in terms of formation of Ala₃ and peptidase activity can be expressed in terms of formation of Ala₂ with Ala₃OMe as the substrate: Ala₃OMe is split into Ala₃+MeOH by the esterase activity of elastase and into Ala₂+Ala-OMe by the peptidase activity.

3. The present method differentiates the esterase or peptidase activities of other enzymes from those of elastase. Ala₃OMe is split to Ala₃, and then to Ala₂+Ala in a stepwise manner by other proteases such as collagenase and pronase E.

4. Trypsin and chymotrypsin enhanced both the peptidase activity and esterase activity of elastase in parallel.

5. The substrate, suc Ala₃NA has been used conventionally to determine elastase activity, and the results with suc Ala₃NA and Ala₃OMe showed a good correlation.

Keywords—elastase; esterase activity; peptidase activity; tri-1-alanine ester; fluorescamine; succinyl tri-1-alanine-*p*-nitroanilide acetate

Elastase (pancreatopeptidase E [EC 3.4.21.11]) activity can be determined with a natural substrate, elastin,^{2,3)} and also with synthetic substrates. Since elastase has two different activities, that is, esterase and peptidase activities, synthetic substrates have been prepared for the determination of each activity. Typical examples are succinyl Ala₃-*p*-nitroanilide (suc Ala₃NA) for peptidase activity⁴⁾ and AcAla₃OMe for esterase activity.⁵⁾

In the present work, the authors investigated the simultaneous determination of both activities using 1-alanyl-1-alanyl-1-alanine methyl ester acetate (Ala₃OMe) as a substrate. A combination of separation on thin-layer chromatographic (TLC) plates and detection with fluorescamine was used to detect the unreacted substrate and enzyme reaction products, and some enzymatic properties of elastase were compared with those of other enzymes.

The present experiments were performed to obtain further information concerning elastase activities.

Materials and Methods

Substrates—1-Alanyl-1-alanyl-1-alanine methyl ester acetate (Ala₃OMe) was obtained from Sigma Chem. Co., St. Louis, Mo., U.S.A., and N-succinyl Ala₃-*p*-nitroanilide (suc Ala₃NA) was obtained from Calbiochem, San Diego, Calif., U.S.A.

1) Location: 2-4-41 Ebara, Shinagawa-ku, Tokyo, 142, Japan.

2) W. Ardelt, S. Ksiezny, and Izabella Nied'zwiecka-Namyslowska, *Anal. Biochem.*, **34**, 180 (1970).

3) I. Banga, J. Balo, and M. Horath, *Biochemistry*, **71**, 544 (1959).

4) J. Bieth, S. Bernard, and C.G. Wermuth, *Biochem. Med.*, **11**, 350 (1974).

5) A. Gertler and T. Hofmann, *Can. J. Biochem.*, **48**, 384 (1970).

Enzymes—Elastase used in the present experiment was from Worthington Biochem. Corp., Freehold, N.J., U.S.A., and the activity was given as 64 U/mg.⁶⁾ The elastase preparation was subjected to polyacrylamide disc gel electrophoresis at pH 4.5 according to Reisfield *et al.*⁷⁾ using 15% gels, and a single band was obtained. This preparation showing elastase I activity,⁸⁾ was used exclusively in the present study. Trypsin was a preparation from Sigma Chemical Co., labeled as Type XII, bovine pancreas, 2× crystallized, 7500 BAEE⁹⁾ U/mg. α -Chymotrypsin was from Sigma Chemical Co., labeled as Type II, bovine pancreas, 3× crystallized, 44 BTEE¹⁰⁾ U/mg. Collagenase was a product of Sigma Chemical Co., prepared from *Clostridium histolyticum*, and labeled as Type II, 250 U/mg. Pronase E was obtained from Kaken Chemical Co., Tokyo (1170000 tyrosine units/g).

Thin-Layer Chromatographic Plates were as follows—Silica gel precoated plates (0.25 mm thick, Merck), and cellulose precoated plates (0.1 mm thick, Merck).

The fluorogenic reagents used were fluorescamine (4-phenylspiro[furan-2(3),1'-phthalan]-3,3-dione (Fluram; Roche, Basel, Switzerland)) and phthaldialdehyde (Merck). A Shimadzu CS-900 dual-wavelength TLC scanner with an adaptor for TLC reflection scanning was used.

Elastase Activity Assay Procedures—The substrate and the products of the elastase reaction were separated on a TLC plate. With Ala₃OMe as a substrate, the esterase activity was expressed in terms of the rate of loss of Ala₃OMe and formation of Ala₃, and peptidase activity was expressed as the rate of formation of Ala₂. A typical enzymatic reaction procedure was as follows. A mixture of 30 μ l of 40 mM Ala₃OMe in sodium borate buffer solution (0.2 M, pH 8.0) and 10 μ l of the enzyme solution was incubated at 37° for 10 min. After the reaction, a 1 μ l aliquot was spotted on a cellulose TLC plate together with known amounts of Ala, Ala₂, Ala₃ and Ala₃OMe as references. It was developed with benzyl alcohol: AcOH: H₂O (4: 1.5: 1) to separate Ala₃OMe, Ala₃, Ala₂, Ala and the enzyme. After drying at 110° for 15 min, the TLC plate was sprayed with 10% triethylamine in dichloromethane and air-dried. The plate was then immersed into a solution of acetone: hexane (1: 4) containing 0.01% fluorescamine for 30 min at room temp, and air-dried. The plate was analyzed with a scanning fluorometer, with excitation at 365 nm and emission at 450 nm.

As shown later, non-enzymatic hydrolysis of the ester occurred, so that the esterase activity in figures is shown after correction for such non-enzymatic degradation of the substrate.

Fluorometric data are shown in terms of relative fluorescent intensity of μ mol.

Results and Discussion

Selection of TLC Plates

Non-enzymatic degradation of the substrate on TLC was examined using silica gel and cellulose TLC plates. The substrate was spotted on cellulose and silica gel TLC plates. Development of the TLC was initiated immediately, and after leaving the spots on TLC plates for 30 min at room temp, they were developed.

It was found that marked non-enzymatic hydrolysis occurred on the silica gel TLC plates, but little on the cellulose TLC plates, so cellulose TLC plates were used in the present experiments to separate and identify the enzymatic reaction products.

Developing Solvent Mixtures

Various solvent mixtures were examined: [*n*-BuOH: AcOH: H₂O], [benzene: dioxane: AcOH: H₂O], [iso-AmOH: AcOH: H₂O], [*tert*-AmOH: AcOH: H₂O], [iso-BuOH: AcOH: H₂O], and [benzyl alcohol: AcOH: H₂O]. It was found that benzyl alcohol: acetic acid: water (4: 1.5: 1) was the most suitable, giving clear spots on cellulose TLC with little tailing. *R_f* values of the enzymatic substrate and products on a cellulose TLC plate developed with the above solvent mixture were 0.83 (Ala₃OMe), 0.66 (Ala₃), 0.61 (Ala₂), 0.42 (Ala), and 0 (elastase).

6) The substrate for the assay was not specified for this preparation, so we determined the activity of each lot in our laboratory. It was found that the activity of the preparation was 7.6×10^4 U/mg; one unit is defined as an increment of 0.001 OD at λ_{410} /min according to the method of Bieth *et al.*⁴⁾

7) R.A. Reisfield, U.J. Lewis, and D.E. Williams, *Nature* (London), **195**, 281 (1962).

8) W. Ardelt, *Biochim. Biophys. Acta*, **341**, 318 (1974).

9) BAEE: assayed with N-benzoyl-L-arginine ethyl ester as a substrate.

10) BTEE: assayed with α -N-benzoyl-L-tyrosine ethyl ester as a substrate.

Detection of the Substrate and Products on TLC

Primary amines could be detected fluorometrically with fluorescamine¹¹⁻¹⁴⁾ or *o*-phthalaldehyde.¹⁵⁾ In the present study, the fluorescence intensities of the substrate and products were compared under the following conditions: the concentrations of Ala₃OMe, Ala₃ and Ala₂ were 1.25×10^{-2} M in 0.2 M sodium borate buffer, pH 8.0; 1 μ l of the solution was spotted on a TLC plate and developed with a mixture of benzyl alcohol: AcOH: H₂O (4: 1.5: 1).

Fluorescence Reagents used were

A) 10% triethylamine in dichloromethane, B) 0.01% fluorescamine in acetone: hexane (1: 4), C) 0.1% *o*-phthalaldehyde and 0.1% mercaptoethanol in acetone, and D) 1% triethylamine in dichloromethane.

Primary amino compounds were detected by fluorescence as follows. After development, the TLC plate was dried at 110° (1) for 15 min, then sprayed with reagent A, air-dried, sprayed with B, and air-dried at room temp for 30 min, (2) for 15 min, then sprayed with A, air-dried, and immersed in B for 30 min, (3) for 30 min, then sprayed with C, and after 5 min, with D, (4) for 30 min, then immersed in C for 5 min, air-dried, and sprayed with D. The intensity and stability of the developed fluorescent spots were compared.

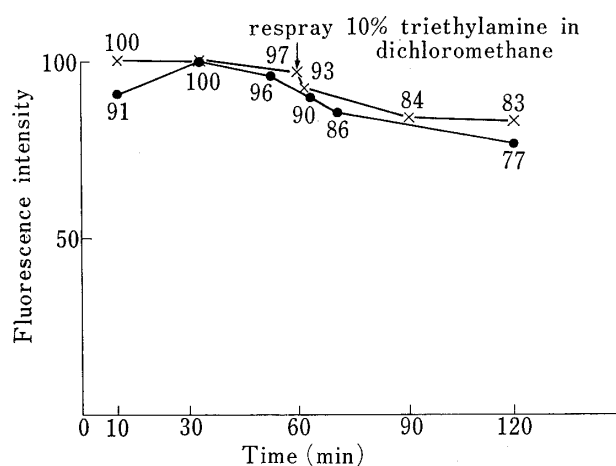


Fig. 1. Time Course of Fluorescent Intensities with Fluorescamine and *o*-Phthalaldehyde as the Fluorescent Reagents

After development and drying at 110°, the procedures described in the text were followed for the detection of the substrate and the products.

—x—: fluorescamine (according to method 2 in the text).
—●—: *o*-phthalaldehyde (according to method 4 in the text).

It was found that the procedures (1) and (3) did not give consistent results, so the procedures (2) and (4) were further examined. Based on the results shown in Fig. 1, it was concluded that the most suitable method was (2).

The use of a stabilizer (10% triethylamine in dichloromethane) was also examined; in method (2), however, it had no effect, even on respraying as described in the literature.¹²⁾ Therefore, in the present study, stabilizer was sprayed only once, immediately before the fluorescence development.

Fluorescamine Application

The effect of solvent composition on the intensity and stability of the developed fluorescent spots on TLC was examined. As shown in Fig. 2, a mixture of acetone: hexane (1: 4) was the best solvent for the present purpose.

Optimum pH for Enzymatic Activity, and Effect of pH on the Degradation of Ala₃OMe

The activities of elastase (peptidase and esterase) were examined at various pH's. It was found that the peptidase activity was maximum at pH 9.0. The apparent esterase activity increased as the pH became higher, but non-enzymatic hydrolysis occurred in the pH region above 9.0 (Fig. 3), so the assay was performed at pH 8.0.

Non-enzymatic degradation of the substrate at pH 8.0 was negligible, when the small blank value was subtracted, on incubation at 37° for up to 20 min (Fig. 4).

11) M. Furlan and E.A. Beck, *J. Chromatog.*, **101**, 244 (1974).

12) A.M. Felix and M.H. Jimenz, *J. Chromatog.*, **89**, 361 (1974).

13) H. Nakamura and J.J. Pisano, *J. Chromatog.*, **121**, 33 (1976).

14) H. Nakamura and J.J. Pisano, *J. Chromatog.*, **121**, 79 (1976).

15) E. Gunner and G. Linderberg, *J. Chromatog.*, **117**, 439 (1976).

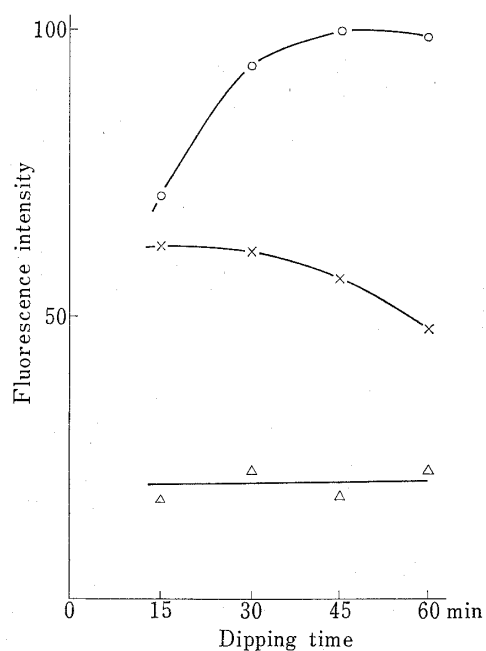


Fig. 2. Effects of Solvents on the Fluorescence Intensity induced by Fluorescamine

—○—, acetone:hexane (1:4).
—×—, hexane.
—△—, acetone.

Time course of the stability and intensity after spotting $1 \mu\text{l}$ of $4.0 \times 10^{-2} \text{ M}$ substrate in $30 \mu\text{l}$ of 0.2 M sodium borate buffer, pH 8.0, on a cellulose TLC plate, drying at 110° for 15 min, spraying 10% triethylamine in dichloromethane, (a stabilizer), then dipping the plate into solutions of 0.01% fluorescamine in various solvents. The fluorescence intensities were determined at indicated time intervals.

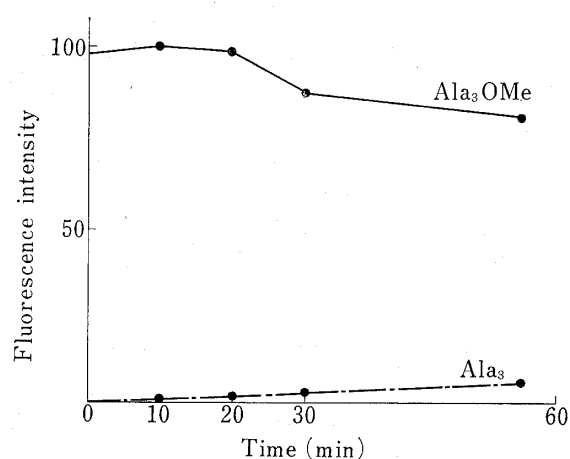


Fig. 4. Time Course of Non-enzymatic Decomposition of the Substrate

Ala₃OMe, 40 mM, was incubated in 0.2 M sodium borate buffer, pH 8.0, for various times at 37° with heat-inactivated elastase.

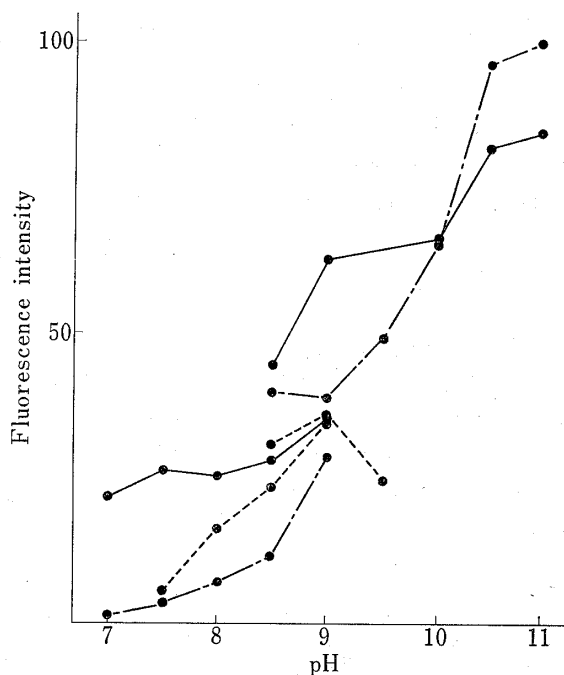


Fig. 3. Optimum pH for Enzymatic Activity and Effect of pH on the Degradation of Ala₃OMe

—●—, esterase activity (Ala₃ formation).
- - -●- - -, peptidase activity (Ala₃ formation).
.....●....., non-enzymatic decomposition (Ala₃ formation).

The elastase activity was examined at various pH's. Ala₃OMe, 40 mM, was incubated in 0.2 M sodium borate buffer, pH 7—9, and 0.2 M carbonate-bicarbonate buffer, pH 8.5—11, for 10 min at 37° with elastase. Esterase and peptidase activities of elastase were estimated as described in "Materials and methods".

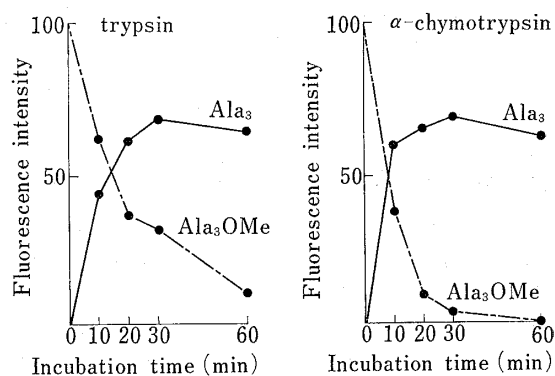


Fig. 5. Esterase Activities of Trypsin and α -Chymotrypsin

A mixture of $30 \mu\text{l}$ of 40 mM Ala₃OMe in 0.2 M sodium borate buffer, pH 8.0, and $10 \mu\text{l}$ of trypsin solution (1500 BAEE units) or α -chymotrypsin solution (8.8 BTEE units) was incubated at 37° .

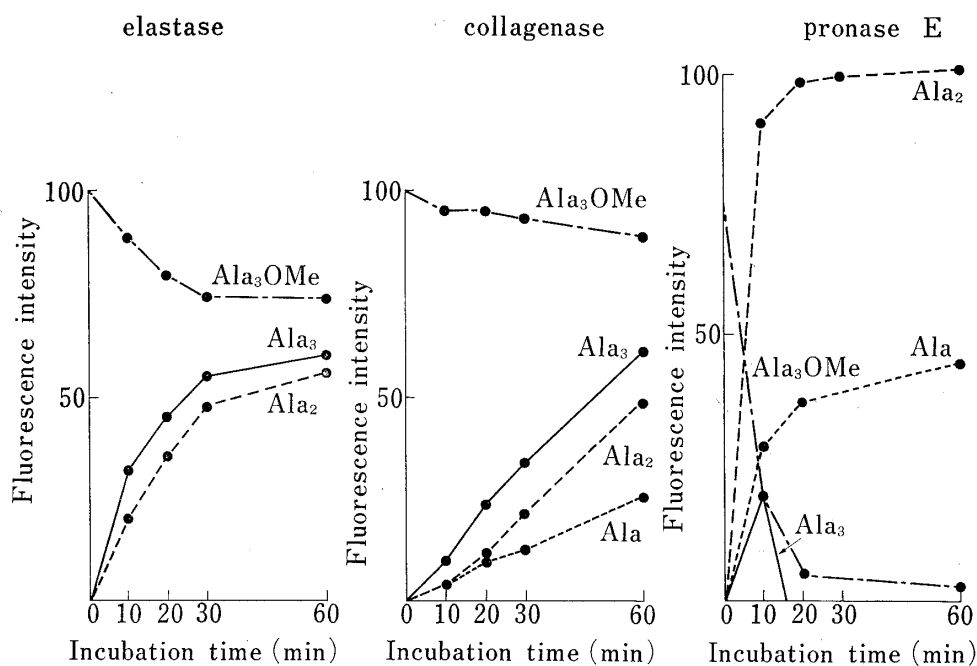


Fig. 6. Comparison of Hydrolytic Activity of Proteases towards Ala₃OMe

A mixture of 30 μ l of 40 mM Ala₃OMe in 0.2 M sodium borate buffer solution, pH 8.0, and 10 μ l of elastase (76 U), collagenase (100 U), or pronase E (29 U) solution was incubated at 37°.

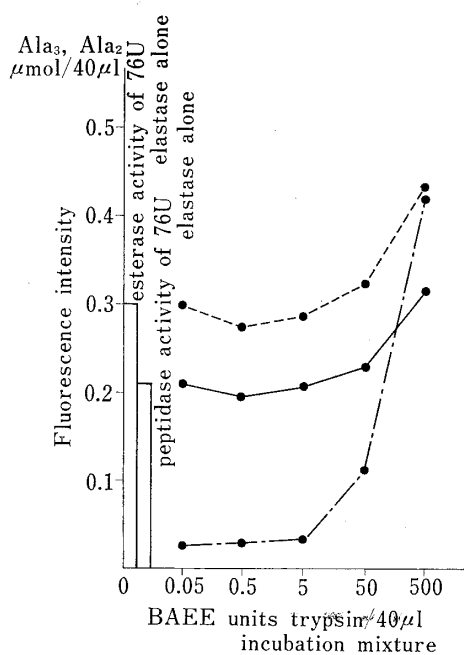


Fig. 7. Potentiation of Elastase Activity by Trypsin

Elastase, 76 units, and various amounts of trypsin were incubated under the conditions described in the text. Vertical bars show esterolytic and peptidolytic activities of elastase alone.

- , esterase activity of elastase (Ala₃ formation).
- - -, peptidase activity of elastase (Ala₂ formation).
- · ·, activity of trypsin alone (esterase, Ala₃ formation).

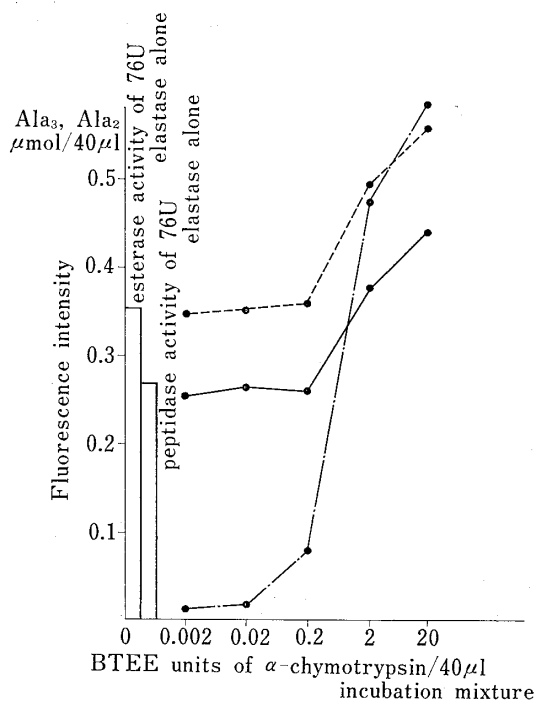


Fig. 8. Potentiation of Elastase Activity by α -Chymotrypsin

Elastase, 76 units, and various amounts of α -chymotrypsin were incubated under the conditions described in the text. Vertical bars show esterolytic and peptidolytic activities of elastase alone.

- , esterase activity of elastase (Ala₃ formation).
- - -, peptidase activity of elastase (Ala₂ formation).
- · ·, activity of α -chymotrypsin alone (esterase, Ala₃ formation).

The effect of buffer composition on enzymatic activity was examined. 0.2 M sodium borate buffer, pH 8.0, was prepared from sodium borate-HCl and boric acid-NaOH. Only esterase activity was exerted in boric acid-NaOH while both esterase and peptidase activities were found in sodium borate-HCl. It appears that chloride is required for the peptidase activity of elastase in the borate buffer system.

Comparison of Elastase and Other Proteases

Esterase activity and peptidase activity towards Ala₃OMe were examined with various proteases. When trypsin or α -chymotrypsin was mixed with Ala₃OMe, only esterase activity was seen, with no peptidase activity (Fig. 5).

When elastase, pronase E or collagenase was incubated with Ala₃OMe, both esterase and peptidase activities were detected, while pronase E or collagenase alone exerted potent peptidase activity, producing alanine (Fig. 6).

With Ala₃OMe as a substrate, elastase has different hydrolytic properties from other proteases; that is, as Gertler *et al.*¹⁶⁾ and Feinstein *et al.*¹⁷⁾ reported, K_m for Ala₃OMe is

TABLE I. Comparison of Elastase Activities towards Ala₃OMe and suc Ala₃NA

a. 76 U elastase		
Peptidase activity	Mean \pm S.D.	CV %
	0.20 \pm 0.03	14.1
b. 38 U elastase		
Peptidase activity	0.11 \pm 0.01	12.7

Peptidase activity:
 $m=2.77$ (Slope of the least-squares regression line).
 $b=0.01$ (Intercept of the least-squares regression line).
 $\gamma=0.98$ (Correlation coefficient).
 $n=12$ (Sample number).

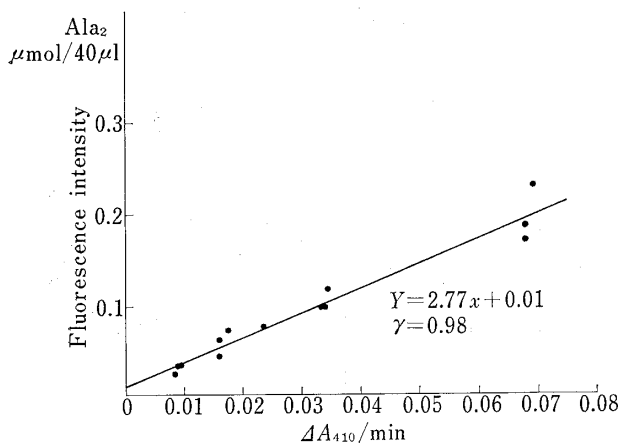


Fig. 9. Comparison of Elastase Activities towards suc Ala₃NA and Ala₃OMe (peptidase activity)

Elastase activities for suc Ala₃NA and Ala₃OMe were determined. The activity of elastase was determined by the method of Bieth⁹⁾ (for suc Ala₃NA) and by the present method (for Ala₃OMe). Four enzyme concentrations were tested in triplicate.

considerably lower than those for Ala₂OMe and for AlaOMe, so the esterase activity of elastase can be estimated by determining the decrease of Ala₃OMe or increase of Ala₃. The peptidase activity of elastase is also characteristic compared to that of other proteases; that is, Ala₂ is produced by the peptidase activity of elastase but Ala is not (Fig. 6). On the other hand, production of Ala₂ as well as Ala is observed when collagenase or pronase is used (Fig. 6).

Thus, the peptidase activity of elastase produces Ala₂+Ala-OMe, in contrast to Ala+Ala₂ produced by other proteases, and it can be determined by estimating the formation of Ala₂.

Rinderknecht *et al.*¹⁸⁾ and Gnosspeli¹⁹⁾ reported that when trypsin and

16) A. Gertler and T. Hofmann, *Can. J. Biochem.*, **48**, 384 (1970).

17) G. Feinstein, A. Kupfer, and Sokolovsky, *Biochim. Biophys. Res. Comm.*, **50**, 1020 (1973).

18) H. Rinderknecht, M.C. Geokas, P. Silverman, Y. Lillard, and B.J. Haverback, *Clin. Chim. Acta*, **19**, 327 (1968).

19) G. Gnosspeli¹⁹⁾, *Anal. Biochem.*, **81**, 315 (1977).

chymotrypsin are added to elastase, their peptidase activities are enhanced. As shown in Figs. 7 and 8, we found that the esterase and peptidase activities were both enhanced in parallel.

At concentrations of Ala₃, Ala₂ and Ala up to 0.5 μ mol/40 μl, when 1 μl of each solution was spotted on a TLC plate, the developed fluorescence intensities were linearly related to concentration, so that elastase activity could be expressed as μ mol of Ala₃ and Ala₂ split from the substrate (Fig. 9). The correlation between the hydrolyses of Ala₃OMe and suc Ala₃NA by elastase was examined (Table I and Fig. 9). The results obtained from Fig. 9 were as follows for peptidase activity: regression line $Y=2.77x+0.01$; correlation coefficient $r=0.98$; $t=14.68$.