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Development of an Agar Diffusion Method to Measure Elastase Inhibition Activity Using Elastin-Congo Red

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Abstract The pancreatic and neutrophil elastases are associated with several illnesses including lung and vascular diseases, various cancers, and pancreatitis. The development of a potent and specific inhibitor to the elastases could lead to new therapies. In this study, an agar diffusion method was modified to include a substrate-dye conjugate (Elastin-Congo red) as a substrate of elastase and an indicator of elastase inhibitory activity. The Elastin-Congo red agar plates consisted of 0.1% Elastin-Congo red and 2.5% agar. The elastase and elastase inhibitors were simultaneously loaded into wells, ultimately resulting in halo formations in which the halo diameter decreased as the concentration of elastase inhibitor increased. The concentration of elastase inhibitor in the samples, therefore, was inversely proportional to the halo diameters. This simplified method provided an excellent correlation with the standard microplate technique, which uses a chromogenic substrate. The concentration of elastase inhibitor obtained from the culture supernatant of a recombinant elastase inhibitor produced by the yeast *Pichia pastoris* was easily determined. This study has established a simple modified and inexpensive agar diffusion method that is potentially useful for the identification, quantification, and screening of new elastase inhibitors.

Key words: Elastin-Congo red, elastase inhibition assay, agar diffusion method, elastase inhibitor

The pancreatic digestive enzyme elastase and circulating neutrophil elastases are destructive agents at sites of inflammation and are known to be associated with several diseases such as pancreatitis, emphysema, cystic fibrosis, bronchitis, asthma, arthritis, psoriasis, and the adult respiratory distress syndrome [14, 17]. Several recent reports also indicate relationships between elastase and cancer [2, 10, 11, 15, 16] and elastase and vascular diseases [1, 13, 18]. These intensively investigated relationships suggest a defined clinical relevance involving the elastases and disease. Therefore, development of potent inhibitors against neutrophil elastases is now recognized as an important emerging field whereby the elastase-related illnesses of inflammation, cancer, and vascular disease should be studied.

At present, the measurement of elastase inhibitory activity that is routinely used involves *N*-Succinyl-Ala-Ala-Ala-*p*-nitroanilide and porcine pancreatic and/or neutrophil elastases as the chromogenic substrate and enzyme, respectively. The inhibition of color development by the elastase inhibitor is measured using a spectrophotometer or a microplate reader. In the present study, an agar diffusion method was assessed in developing a simpler technique to quantify elastase inhibition activity, by using the substrate-dye conjugate (Elastin-Congo red) incorporated into agar as the substrate and guamerin [4] as the elastase inhibitor.

This study used recombinant guamerin as an elastase inhibitor. Guamerin was originally identified in the Korean leech (*Hirudo nipponia*), and consisted of a 57 amino acid peptide with 10 cysteine residues. *H. nipponia* guamerin was characterized by potent inhibitory activity and specificity against human neutrophil and pancreatic elastases [4]. The test samples containing guamerin employed in this study were obtained from the culture supernatant of the yeast *Pichia pastoris*, in which the gene expressing guamerin had been integrated into chromosomal DNA. To obtain the guamerin standard, the cultivation and purification procedures, previously described in detail [5–9, 12], were adopted.

Porcine pancreatic elastase solution (E1250, protein concentration, 0.350 mg/ml; specific activity, 7.8 units/mg-protein, Sigma, U.S.A.) and Elastin-Congo red (Sigma, U.S.A.) were used as enzyme and substrate, respectively. Elastin-Congo red is a substrate-dye conjugate, in which the cleavage of Congo-Red from the conjugate indicates the elastase activity. In the modified agar diffusion assay, 0.1% Elastin-Congo red and 2.5% agar (Difco, U.S.A.) were melted simultaneously in 15 ml of a 0.2 M Tris-HCl

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buffer (pH 8.2). The agar solution was allowed to solidify in a plastic petri dish (87×15 mm); thereafter, wells (diameter =5.6 mm) for sample loading were manually created using a cork borer.

The test samples (10 μl) and enzyme solution (10 μl) were mixed in a microcentrifuge tube and incubated for 10 min at room temperature. Ten μl of the incubated test samples were then loaded into a sample well in the Elastin-Congo red agar plate. The agar plates were incubated for either 12 h or 24 h at 37°C; any subsequently formed halo diameters around each well were measured using electronic calipers (Mitutoyo Co., Japan).

The halo diameters were normalized by dividing these by the maximum halo diameter and then those were represented as the relative diameter. Standard calibration curves for the guamerin concentration vs. the relative halo diameter were generated by taking standard samples of the recombinant guamerin (10 mg/ml) through a series of two-fold dilution steps. The amount of recombinant guamerin in the *P. pastoris* culture supernatants was calculated from the calibration curve.

Porcine pancreatic elastase (E7885, Sigma, U.S.A.) and *N*-Succinyl Ala-Ala-Ala-*p*-nitroanilide (Sigma, U.S.A.) were used as enzyme and chromogenic substrate, respectively. The digestive reaction was performed under buffered conditions (0.05 M Tris-HCl, pH 8.2) using 96-well microplates to which 50 μl of buffer solution containing the elastase (1.0 unit/ml) and 50 μl of sample solution (culture supernatant) were added, followed by thorough mixing and the addition of 150 μl of 0.5 mM substrate. After a 10-min incubation at room temperature, the absorbance was measured at 415 nm using a microplate reader (BioRad, U.S.A.). The amount of elastase inhibitor in test samples was estimated from the concentration of the purified recombinant guamerin solution (IC_{50}) that inhibited enzyme activity at the 50%-inhibition level in this assay system. When necessary, the test samples were diluted properly until their inhibitory activities reached the 50%-inhibition level. The extent of dilution (DF_{50}) of test sample required to inhibit the enzyme activity at a 50%-inhibition level was obtained from the plot of the percent inhibition of enzyme activity vs. the extent of sample dilution. The amount of recombinant guamerin in the culture supernatants could be calculated by multiplying the IC_{50} and DF_{50}.

Elastin-Congo red is an insoluble conjugate of insoluble protein (elastin) and dye (Congo red). When elastase

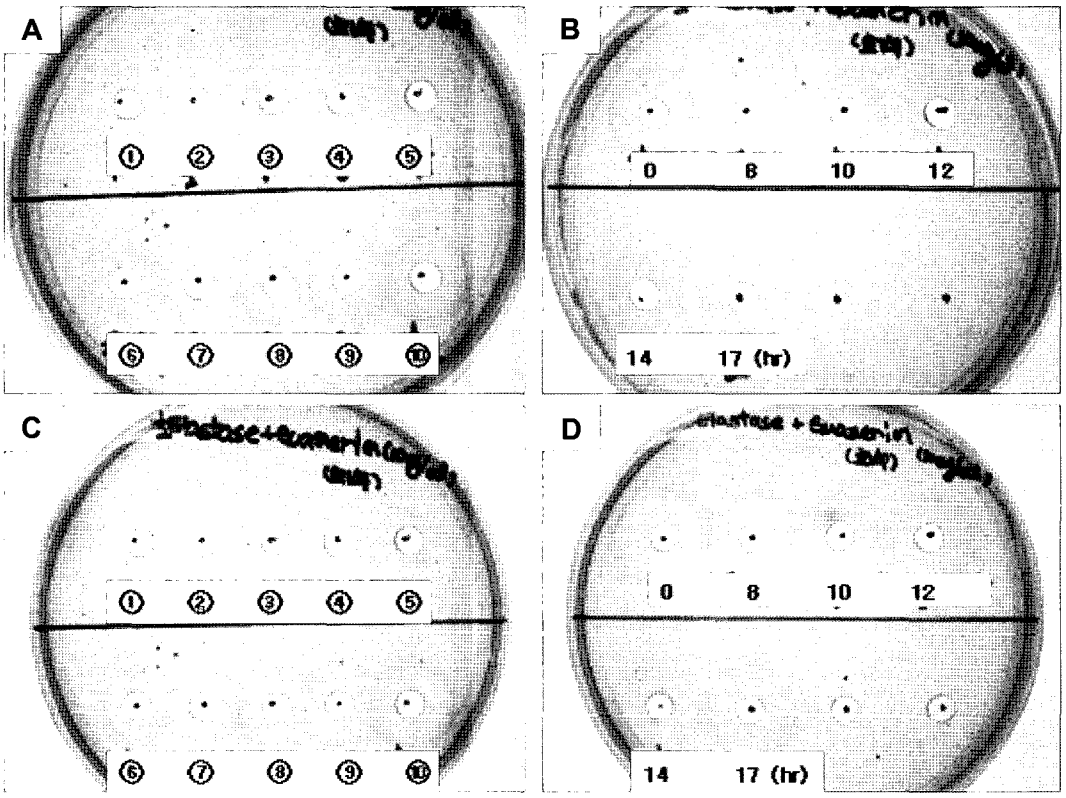

Fig. 1. Agar diffusion method using Elastin-Congo red for the elastase inhibition assay. The protein concentration of porcine pancreatic elastase was set as 0.175 mg/ml (specific activity=7.8 units/mg-protein).

A, **C**. Standard recombinant guamerin. Guamerin concentrations of loading samples (from ① to ⑩) were 10.0, 5.0, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, and 0.019 mg/ml, respectively; an initial sample of guamerin (10 mg/ml) was serially diluted in two-fold steps. **B**, **D**. Culture supernatants; 0, 8, 10, 12, 14, 17 h samples. **A**, **B**. 12-h incubation at 37°C. **C**, **D**. 24-h incubation at 37°C.

hydrolyzes the chemical bond between elastin and the dye, the dye is released and the color of the reaction mixture changes to red in a basic pH environment. The agar plates that contain Elastin-Congo red initially were red in color. Small insoluble aggregates of Elastin-Congo red in the agar plates were visible to the naked eye. If elastase was present in the well, the area surrounding the well was altered to a reddish clear halo as a result of the radial diffusion of elastase and the subsequent hydrolysis of the Elastin-Congo red in the agar. To detect elastase inhibitory activity, a mixture of both elastase and a suspected elastase inhibitor were preincubated for 10 min at room temperature prior to loading into the well. Should there be an increase in the concentration of elastase inhibitor, the halo diameter would be decreased because of inhibition of the elastase activity.

As shown in Figs. 1A and 1C, the decrease in concentration of elastase inhibitor in test samples resulted in an increase in halo diameter. Increasing the incubation time from 12 h to 24 h did not affect the halo diameters. The culture supernatant of guamerin produced by *Pichia pastoris* was also loaded into wells in the Elastin-Congo red agar plates (Figs. 1B and 1D). As the induction time for the expression of the recombinant guamerin elapsed, the halo diameters decreased, reflecting the increased accumulation of the guamerin into the yeast culture medium. The test sample incubation time (12 h or 24 h) in this study was also without affect on halo diameters. In an attempt to measure the concentration of elastase inhibition in the test samples, the relationship between the relative halo diameter and the concentration of elastase inhibitor were plotted, as shown in Fig. 2. The data sets (●) of Figs. 2C and 2D were derived from Figs. 1A and 1C. The remaining plots in Fig. 2 represent the relationships obtained with the concentrations of elastase set at 0.350 or 0.175 mg/ml and incubation intervals of 12 h or 24 h. The four plots of Fig. 2 demonstrate the linear relationship for the relative halo diameter vs. the concentration of elastase inhibitor. These results indicate that Elastin-Congo red agar plates can be used to measure the concentration of elastase inhibitor until 0.6 mg/ml. In this measurement system, if the elastase enzyme solution is too dilute for the agar diffusion assay, the resulting halo diameter is minimal and not suitable for measure (data not shown). Furthermore, if the incubation time exceeded

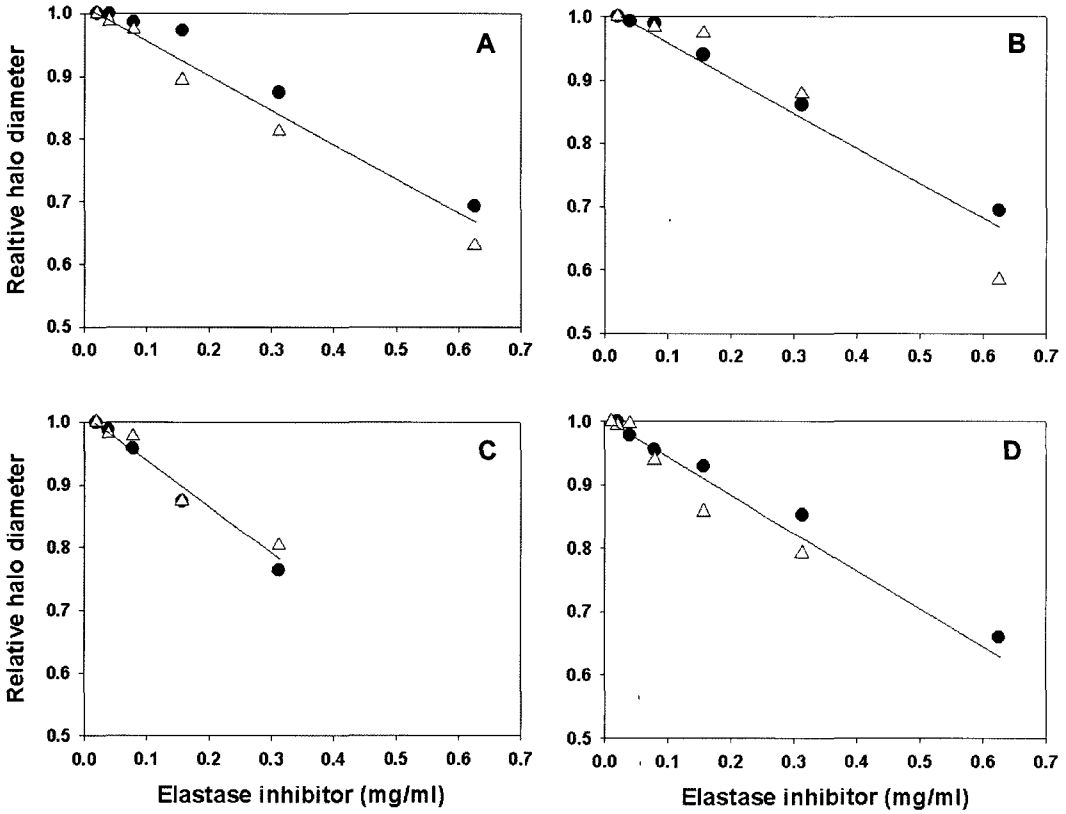

Fig. 2. Relationships between the relative halo diameter and the concentration of elastase inhibitor.
Data (●) of **C** and **D** were obtained from Fig. 1. Other data (△) were obtained following the same methods as described in Fig. 1. Data (●, △) of **A** and **B** were obtained from a duplicate experiment. The protein concentrations of porcine pancreatic elastase solution used in **A** and **B** were 0.35 mg/ml and those in **C** and **D** were 0.175 mg/ml. Incubation periods of **A** and **C** were 12 h and those of **C** and **D** were 24 h. The specific activity of the enzyme solution was 7.8 units/mg-protein.

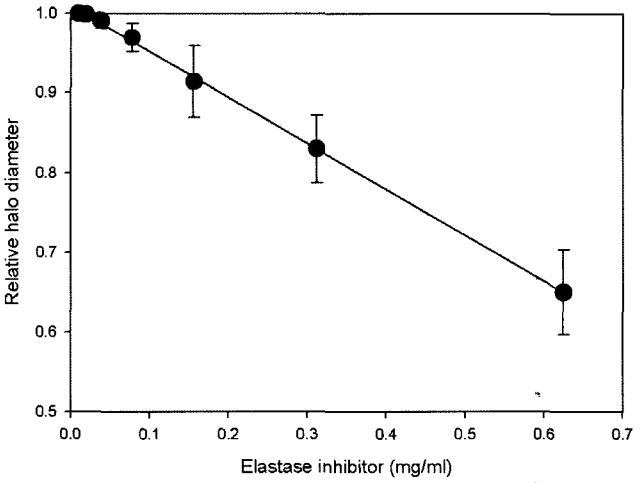

Fig. 3. Standard curve for the relative halo diameter vs. elastase inhibitor content.
The solid line was obtained from a linear regression (Y=−0.576X+1.00, r^2=0.999) of the average data point of each elastase inhibitor concentration (0.625, 0.312, 0.156, 0.078, 0.039, and 0.019 mg/ml). The data sets were taken from Fig. 2. Except for the data at a concentration of 0.625 mg/ml (n=5), the eight data sets (n=8) at each concentration were used for the linear regression analysis. The standard deviation at each concentration is shown as error bars.

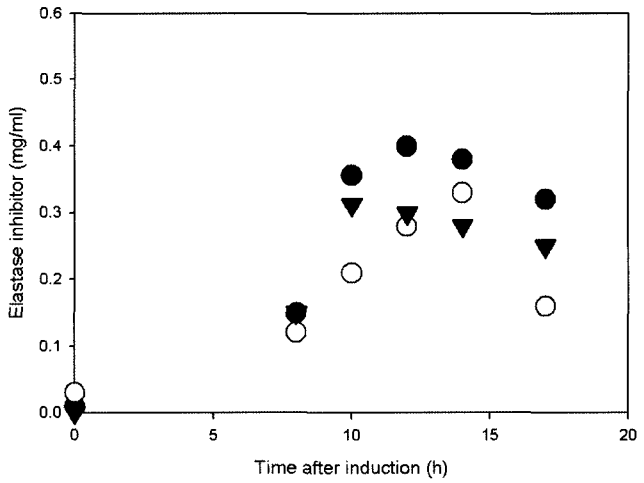

Fig. 4. Measurement of elastase inhibition activity using the agar diffusion method (●, ○) and microplate method (▼).
The data expressed by ● and ○ differed according to incubation intervals, 12 h and 24 h, respectively; data were taken from Figs. 1B and 1D.

24 h, the outer limits of the halo became indistinct (data not shown). Therefore, the optimal assay conditions for the quantification of elastase inhibitors as developed in this study are described in Fig. 2.

Linear regression analysis of the relationship between relative halo diameter and concentration of elastase inhibitor yielded a straight line (Fig. 3). This regression line could be used as a standard curve to determine the concentration of elastase inhibitors in test samples. Thus, by using this Elastin-Congo red agar plate technique, we could quantify the concentration of elastase inhibitor in the culture supernatant from genetically modified *Pichia pastoris*. To compare these results with that of the established method, the concentration of elastase inhibitor was also measured using the microplate method involving chromogenic substrate. The value obtained from same samples using Elastin-Congo red agar diffusion (Figs. 1B and 1D) correlated well with the standard microplate method (Fig. 4).

These results suggest that our agar plate diffusion method for quantifying test sample content of elastase inhibitors could be a routinely useful tool because of its simplicity and inexpens, since no costly instrumentation is required. As the elastase activity in human tissues and sera is increasingly recognized to be a factor involved in many human diseases, readily available methods of detecting enzyme inhibition assume greater importance. Although the alternative elastase inhibition assay method is commercialized (Elastin Products Company, U.S.A.) [3] and has been available since 2004, we have provided basic evidence for a method using Elastin-Congo red to be a simple tool for measuring elastase inhibition activity and for the screening of new elastase inhibitory agents.

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