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Human Neutrophil Elastase: Rapid Purification, Metal binding Stoichiometry and Modulation of the Activity by Chelating Agents

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ABSTRACT

Neutrophil elastases were purified by a three step procedure consiting of one Sephadex G-75 and two HPLC elutions. The elastases cross-reacted with antibodies to human neutrophil elastase. Three bands with molecular weights between 26,000 and 29,700 were observed by gel electrophoresis. At each stage of purification the quantity of Zn increased, reaching molar ratio of 2:1 with elastase in the most purified samples. Calcium content. was seletively elevated during the earlier stages of purification but decreased to a ratio of 0.25 to 1 with elastase at the final step of purfication. Neutrophil elastase could be inhibited by EDTA, EGTA and 1,10-phenanthroline. EGTA inhbition was noncompetitive inhibition and reversible only if the time of preincubation was relatively short, indicating the instability of the apoenzyme. The concentration of chelator required to show significant inhibition of elastase was also dependent upon the stage of purity and the ionic strength of the reaction mixture. Inhibition by EGTA, followed by the removal of EGTA, could be reversed by Zn. In the presence of EGTA the enzyme could be returened to full activity by the addition of Zn, Mn and Ca, but not Mg or Na. All of the above evidence strongly supports human neturophil elastase could be a metalloenzyme as well as a serine protease.

Key Words: Neutrophil, Elastase, Metal ion, Chelaters
Abbreviations: SANA: N-Succinyl-L-Alanine-L-Alanine-L-Alaniese-p-Nitroanilide

INTRODUCTION

Elastases (3.4.21.11) are a group of enzymes characterized by their ability to hydrolyze insoluble elastin (Beith, 1978). Activity of these enzymes is not limited to elastin, however as many other proteins, including casein, hemoglobin and collagen, are also susceptible to degradtion (Starkey, 1977). Leukocyte elastases are active participants in the acute phase of inflammation (Starkey, 1980) and have been implicated in a number of diseases including atherosclerosis (Bieth, 1978), rheumatoid arthritis (Janoff, 1972b) and emphysema (Janoff, *et al.*, 1979). It is therefore interest to investigate the fundamental characteristics and the factors which are capable of inhibiting the activity of these enzymes.

Althouth the PMN and pancreatic elastases are generaly classified as serine proteinases there are reports indicating that EDTA can inhibit pancreatic elastases but human neutrophil elstases at concentration of 5 mg/ml (Starkey & Barrett, 1976). Crystallized preparations of pancreatic elastase have been reported to contain calcium and zinc (Lewis *et al.*, 1956). We have reported that EGTA is also an efficient inhibitor of PMN elastase and that inhibition can be reversed by the addition of Ca^{++} but not Mg^{++} (Kang, 1985). Sensitivity to EDTA has also been shown for the macrophage elastases (Gordon & Werb, 1976; Banda & Werb, 1981). It has been reported that calcium is required for the activity of the mouse macrophage elastase and this enzyme has been designated as a metalloproteinase (Banda & Werb,

1981). The microbial elastase derived from Flavobacterium immotum is completely inhibited by 1, 10-phenanthroline (Ozaki & Shio, 1975).

It is not known whether the metal ion chelators inhibit the human neutrophil elastases by directly binding to the enzyme or indirectly through removal of a metal ion constituent of the enzyme. The elucidation of the mechanism of inhibition produced by these chelators will provide important information on alternative methods of regulation of this serine proteinase.

Toward that end we provide data on the characteristics of inhibition of human PMN elastase by EDTA, EGTA and 1,10-phenanthroline and some preliminary evidence which suggest that zinc and calcium ions are tightly bound to elastase. Specially, Zn ion may be involved in both catalytic and structural component of human neutrophil elastase. But Ca ion may involves only for catalytic activities.

MATERIALS AND METHODS

Preparation of the crude leukocyte extract

Human leukocytes were spearated from plasma and red blood cells by the method of Kang (1985).

Purification of human leukocyte elastase

a) Sephadex G-75 chromatography: Aliquots (usually 8-16 ml) of the crude leukocyte extract were first chromatographed, at 4℃, through a 2.5×100 cm column of Sephadex G-75 equilibrated with 0.05 M Tris-HCl buffer, pH 7.3, contanaining 0.2 M NaCl and 0.005 M $CaCl_2$ and 0.1% Brij 35. Fractions were assayed for protein concentration (Lowry, Rosebrough, Farr & Randell, 1951), cathepsin G (Starkey, 1977), collagenase (Harris & Vater, 1982), and elastase activity using Suc-ala-ala-ala-pnitroanilide (SANA) and insoluble, H^3-labelled elastin (Takahashi, *et al.*, 1973). The fractions demonstrating elastase activity, usually containing some catheptic but no collagenolytic activity, were pooled and concentrated to one-tenth volume by ultrafiltration through an Amicon PM 10 membrane.

b) high performance liquied chromatography; affinity separation: For this first HPLC separation a BioSil TSK 250 column (BioRad) was equilibrated with 0.01 M of sodium acetate, pH 5.5, containing 0.02 M NaCl, and eluted at a constant flow rate of 1.0 ml/minute using a Beckman Model 322 HPLC system. Eight to 16 ml of concentrated elastases from Sephadex G-75 chromatography was applied to the TSK gel column by the repeated injection of 1.0 ml of the smaple separated by ten minute intervals. Absorbance of the eluate was monitored with a Beckman UV detector at a fixed wavelength of 280 nm.

The column was continually washed with buffer until all unbound protein was eluted then followed by a gradient of sodium chloride from 0.02 M to 1.0 M, in 10 mM sodium acetate at pH 5.5. The eluate was fractionated into test tubes and every other fraction from the column was assayed for elastase and cathepsin G as described. The fractions of peak elastase activity, which eluted in the middle of the salt gradient and were free of catheptic activity, were pooled, adjusted to pH 7.3 and concentrated.

c) High performance liquied chromatography; gel filtration separation: 0.2 ml of the concentrated elastase dervied from the previous HPLC separation was applied to BioSil TSK 250 (or TSK 3000 SW) column equilibrated in 0.05 M Tris-Cl, pH 7.3, containing 1.0 M NaCl. The column was eluted at a constant flow rate of 1.0 ml/minute at room temperature. Protein content and elastase activity were measured as described. The fractions demonstrating elastase activity were pooled and concentrated to 1/10 of the original volume by ultrafiltration using an Amicon UM 2 membrance.

Enzyme assay

a) Elastase assay with SANA: Elastase assay was performed by modification of Starkey & Barrett (1976b), i.e. elastases were incubated in 0.2 ml of reaction medium containing 50 mM Tris-Cl, pH 7.3, 150 mM NaCl and 5 mM $CaCl_2$ at 23℃ for 10-20 minutes. The reaction was started by adding a stock solution of SANA disolved in reaction medium and incubated at 23℃ or 37℃ for defined time. The quantity of liberated nitroaniline was determied by using Titertek Multiskan spectrophotometer (Finlab Model 310 C, HelsinkI, Finland) at 410 nm of wavelength or by a direct recording the absorbance with spectrophotometer (Gilford/Backman) in a temperature controlled cuvettes.

b) Elastase assay with insolulble elastin: Radioactive elastin was prepared by chemical reduction of desmosine and isodesmosine crosslinks of bovine nuchal ligament elastin (Takahashi *et*

al., 1973). Specific activity of radioactive elastin was 1000-1500 cpm/ug. In a series reactions, elastases was mixed with radioactive elastin (25,000 -100,000 cpm) in 0.25 ml of the reaction medium. The reactions were carried out at 37℃ with continous agitation and stopped by adding 100μl of 0.5 M acetic acid and centrifuged at 10,000 g for 10 minutes. The radioactivity relased into the supernatant of the reaction mixture was used for measuring the enzyme activity.

c) Cathepsin G assay: The standard incubation mixture contained an aliquot of elastase, 100 mM Tris-Cl, pH 7.3, 0.1% Brij 35 and 4 mM benzoyl-DL-phenylalanine napthyl ester (freshly diosolved in dimethylsulfoxide) in a final volume of 0.4 ml (modified from Starkey, 1977). The reaction was carried out at 50℃ for 20 minutes and stopped by the addition of 0.6 ml of coupling reagent prepared as follows: 0.1 ml of 0.2 M $NaNO_2$ was added to 1 ml base stock solution on ice, followed by dilution to 50 ml with 4% (v/v) Brij 35 in water. The base stock soultion contained 225 mg of 4-amino-2,3-dimethylazobenzene disolved in 50 ml ethanol and 30 ml of 1 M HCl with stirring, then diluted to 100 ml with water (stored at 4℃). The color was allowed to develop at room temperature. After 5 minutes the reaction mixture was centrifuged and absorbance was read at 520 nm.

Electrophoresis

At vaious stages in the purification, the elastases preparation was examined by polyacrylamide gel electrophoresis (Laemmli, 1970). When required, samples were dialyzed against HPLC grade water and then lyophilized before electrophoresis.

Elemental analysis

Elemental composition was assayed by energy dispersive X-ray fluorescence (Sky-Peck & Joseph, 1981). Samples to be analyzed were air-dried on to a formvar coated surface in laminar flow hood. The specimens were then placed in a vacuum chamber fluorometer and the X-ray spectral response to 0 to 22 Kev exciatation (15 milliamps, 35 kilovolts) was measured and compared to standard elements.

Immunological studies

Crude neutrophil extracts and the pooled elastase activity peaks from the various purification stages were examined for their reactivity with antibodies to human neutrophil elastase (generous gifts from Dr. Carlo Mainardi, Rutgers Medical school and Dr. Sigfried Neumann, E. Merk, Darmstadt, FDR) in Ouchterlony diffusion and ELISA assays. The antisera was shown not to be cross-reactive with human monocyte elastase. Antisera was placed in the center well of an agarose immunodiffusion assay plate with surrounding wells filled with either crude elastase or elastases in various stages of purification, cathepsin G or collagenase. Diffusion was allowed to proceed freely for 18 hours at 37℃ in a temperature and humidity controlled chamber.

Infuluences of Chelating Agents

Crude leukocyte extracts or fractions derived from the various chromatographic procedures were incubated with Tris/EDTA, Tris/EGTA or 1,10-phenanthroline, pH 7.3, for ten minutes to 18 hours. Unless otherwise specified, the routine incubation time was one hour. To best demonstrate inhibition elastase should be dissolved in, or have been dialysed against dilute buffer, e.g. 50 mM Tris-HCl, pH 7.3, NaCl may be added but as the concentration of salt is increased, the subsequent increase in activity masks the detection of inhibition. Tris/EDTA and Tris/EGTA were prepared in stock solutions of 0.1 M and buffered to pH 7.0 with solid Tris. 1,10-Phenanthroline was dissolved at a concentration of 10 mM in water or at a concentration of 100 mM in 20% ethanol. To the mixture of enzyme, chelator and Tris buffer to equalize the ionic strengths in all the reaction wells and, after the desired preincubation time, a stock solution of either SANA or ^{3}H-elastin was then added. The final substrate concentration was between 0.5 and 5 mM depending on the nature of the experiment but concentrations at or below 1 mM are more favorable to the detection of inhibition. Due to the apparent instability of the apoenzyme (the elastase without metal) the reversibility of the inhibition was determined by two methods. First, various amounts of elastase (2.7 ug to 14 ug) were preincubated in 0.2 ml of reaction medium containing 50 mM Tris-HCl, pH 7.3, 150 mM NaCl and 5 mM $CaCl_2$, with or without 0.01-0.05 M Tris/EGTA for 30 minutes to 18 hours. The reactions were initiated by adding SANA or tritium labeled elastin (a final concentration of 5 -10 ug of elastin) and incubated at 37℃. Affer a

preincubation period in the presence of Tris/EGTA, buffered $CaCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$ or NaCl was added to a final concentration higher than that of the EGTA. Released p-nitroaniline or solubilized ^{3}H-elastin peptides were measured as described. Second, HPLC purified elastase dialysed against in 50 mM Tris-HCl, pH 7.3, was inhibited by the addition of 0.1 M Tris/EGTA (final concentration of 50 mM) for 15 to 30 minutes until enzyme activity was reduced at least to 80%. The appropriate time was established on each batch of HPLC purified enzyme used for these experiments. The mixture was rapidly passed through a PD-10 column (prepacked G-25 Sephadex), equilibrated in 50 mM Tris buffer, pH 7.3, to separate the enzyme from the Tris/EGTA. The Vo of the column was eluted directly into tubes containing 0.1 M NaCl or 0.1 M NaCl and 0.002 to 0.02 M ZnCl or $CaCl_2$. A stock solution of SANA was added to give a final substrate concentration of 0.005 M and color yield was determined after 15 minutes of incubabion at 37℃.

RESULTS

Purification of human leukocyte elastase

Sephadex G-75 provided a satisfactory method for the initial separation of the majority of proteins and enzymes in the leukocyte extract from elastase (Fig. 1). In occasional preparations, collagenase and cathepsin G overlapped the elastase peak. Howewver, with the addition of 0.1% Brij 35 to the bufer, separation of these enzymes reached optimal levels. Elastase was found in a broad peak of activity with both the synthetic and natural substrate from the majority of proteins and all of the cathepsin G activity in the G-75 pool of enzyme activity was accomplished with the first HPLC separation (Fig. 2). Arrow 1 indicates the start of repeated injections of the pool of concentrated elastase from the previous Sephadex G-75 gel filtration column. At this time the full-scale absorbance, at 280 nm, was 3.2. More than 60 times the recommended maxium volume of the sample (8-16 ml) could be loaded onto the 250 SW column. After the last injection, and when the baseline had returned to the absorbance of the buffer, the full scale absorption was changed to 0.08 and the salt gradient begun (arrow 2). Elastase activity eluted in a variable number of peaks depending on the slope of the salt gradient, along with a protein peak that was usually confined to the early region of the elastase activity, eluting at NaCl concentrations between 0.4 and 0.6 M. HPLC "affinity"

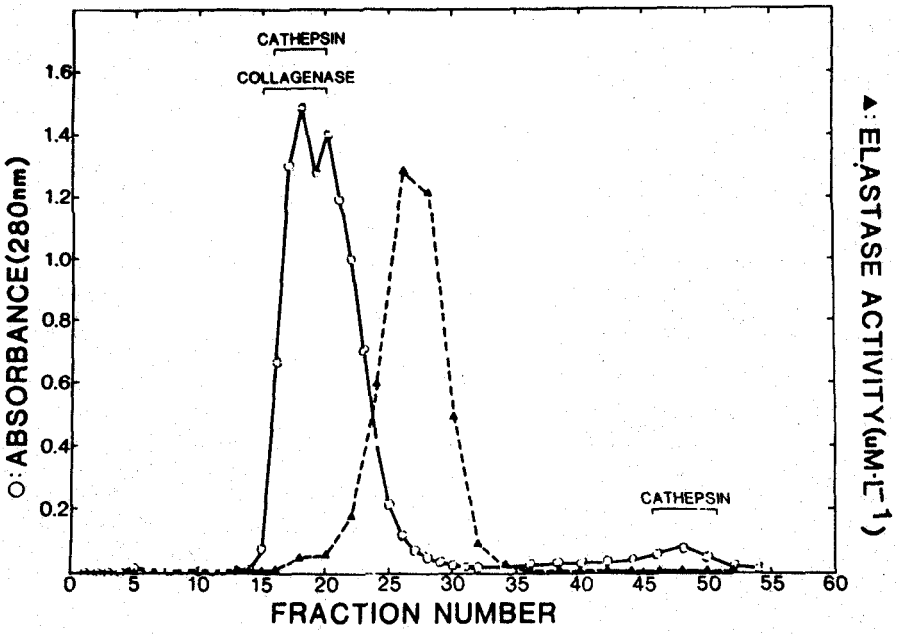

Fig. 1. Chromatography of human leukocyte extract on Sephadex G-75 ; The column (2.5 x 90 cm) was equilibrated with 50 mM of Tris-Cl, pH 7.3, containing 0.2 M NaCl and 5 mM of $CaCl_2$ at 4°C. 5 ml of leukocyte extract was loaded and eluted at a flow rate of 24 ml/hour. O---O : protein ; ▲ - - - ▲ : elastase.

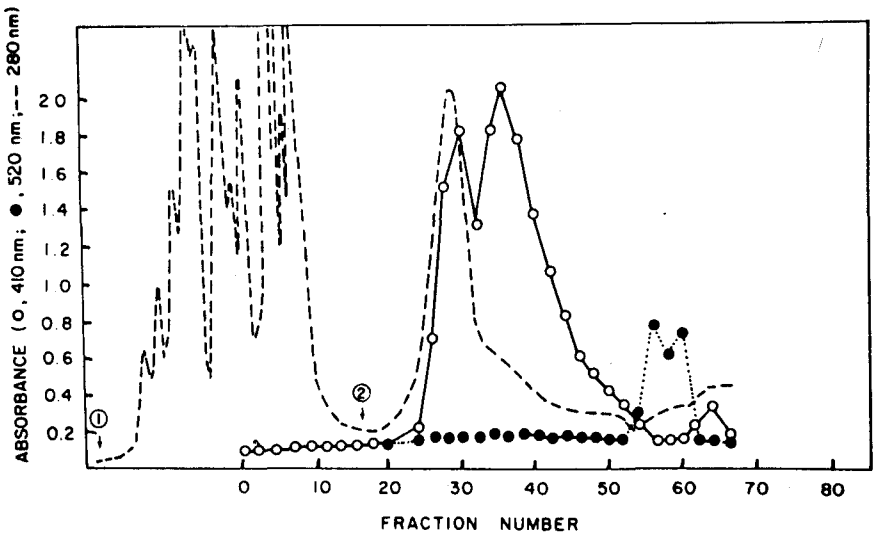

Fig. 2. Purification of elastase by HPLC (affinity method) ; The TSK 250 column was equilibrated with sodium acetate (pH 5.5) containing 20 mM of NaCl and 0.1 % of Brij-35 at a flow rate of 1 ml/minute at 23°C. 10 ml of concentrated, Sephadex G-75 derived elastase was loaded, by repeated injection of 1 ml samples (arrow 1). After the absobance returned to baseline, NaCl gradient (0.02 – 1 M) was started (arrow 2). (-----) : protein at 280 nm : (○ ----- ○) ; elastase ; (● ----- ●) ; cathepsin G.

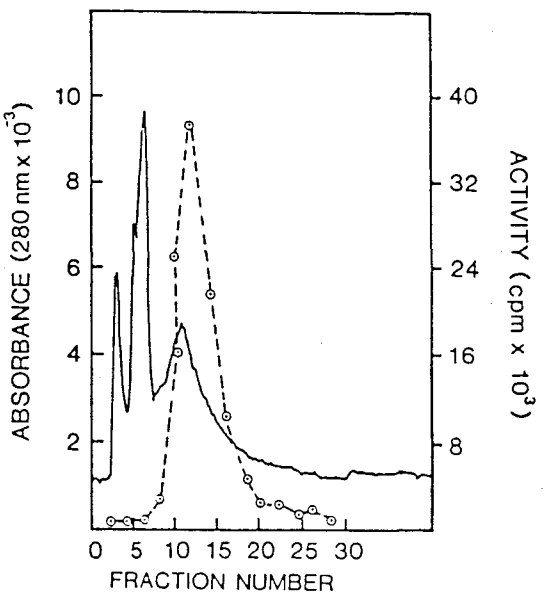

Fig. 3. HPLC purification of elastase (gel filtration) : The TSK 250 SW column was equilibrated with 10 mM Tris-Cl, pH 7.3, containing 1.0 M NaCl. Concentrated elastase from previous purification was dialyzed against the sodium acetate buffer, loaded onto the column and eluted at a flow rate of 1 ml/minute at 23°C. The column was monitored continuously at a wavelength of 280 nm. (○---- ○) : elastase.

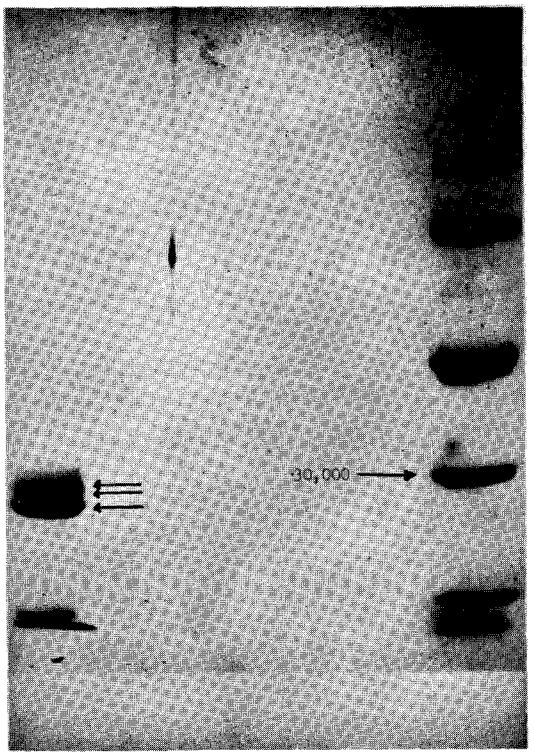

Fig. 4. Polyacrylamide Gel Electrophoresis : Lane 1 : elastase ; Lane 2 : protein standard. The gel was stained with Coomassie Blue and destained with 7% acetic acid.

Table 1. Elastase activities

Step	Total protein (mg)	Specific activity (μM/mg· min)	Total activity (μM/min)	Yield (%)	Fold purity
Crude	272	.53	14.4	100	1
G-75	3.6	3.8	13.6	94	7
HPLC	0.09	64.5	5.4	38	120
HPLC*	0.016	294	4.7	33	555

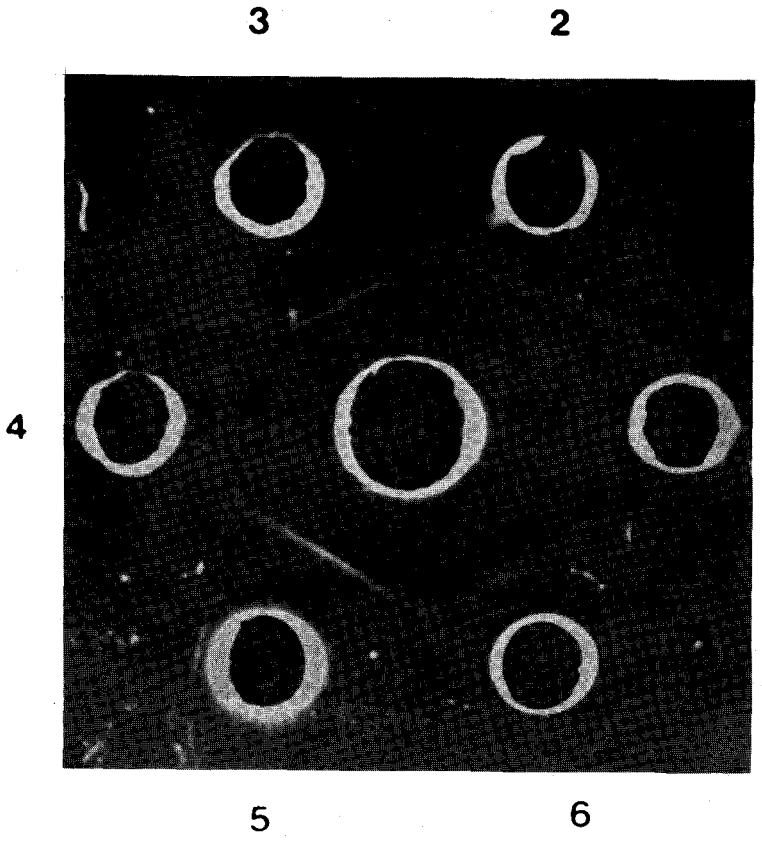

Fig. 5. Ouchteriony immunodiffusion assay : Antibody to elastase was placed in the center well and surrounded by elastase preparations at various stages of purification, collagenase and cathepsin G ; 1 : collagenase ; 2 : cathepsin G ; 3 : first HPLC purified elastase ; 4 : second HPLC purified elastase ; 5 : Sephadex G-75 purified sample ; 6 : crude extract.

purified elastase was reapplied to the TSK 250 SW column to remove the remaining contaminant proteins. Because of the high ionic strength employed, 1.0 M NaCl in 10 mM Tris-Cl buffer, binding of these proteins to the column was prevented and separation was achieved by the molecuar sieving property of the column (Fig. 3). Assays of each of the column fractions with SANA and ^{3}H-elastin demonstrates a single symetrical peak of activity with each substrate. No resolution into the individual enzyme species is seen since the four elastases have similar molecular weights (Fig. 3).

Biochemical characteristics

To examine the purity of the enzyme at this final stage, polyacrylamide gel electrophoresis in the presence of SDS was performed and the migrations of the enzymes were compared to globular

Table 2. Elemental analysis

Element	Mass ratio (μg/g protein)				
	Crude extract	G-75	1st HPLC	2nd HPLC	2nd HPLC+ EGTA dialysis
Ca	184	776	5182	665	103
Mn	3.4	0.9	1.8	2.0	1.5
Fe	1270	485	704	35	24.6
Zn	68	104	5806	5640	13.0
Pb	11.9	7.6	221.2	5.5	3.9

protien standards (Fig. 4). The molecular weights of the elastase species resolved on polyacrylamide gels were 29,700, 28,200 and 26,400. Enzyme activity at the various stages of purification is shown in table 1. Total fold purificaton was greater than 550 with a yield of 30%. The Km for elastase with this substrate was 1 ± 0.05 mM. The Ouchterlony immunodifusion gel assay showed immunological cross-reactivity between neutrophil elastase antibodies and crude leukocyte extract, G-75 fractionated material and the HPLC purified elastase. No cross-reactivity with neutrophil cathepsin G or collagenase was detected (Fig. 5). The results of elemental analysis are shown in Table 2. The quantity of Fe^{++} was decreased as the purification proceeded to the most homogenous preparation of elastase. On the other hand, quantities of Zn^{++} and Ca^{++} were significantly elevated by the final stage of purification. The quantity of Ca^{++}), however, dropped at the last stage of purification. The molar ratio of Zn^{++} to elastase was 2.2 to 1. The molar ratio of calcium to elastase was 0.25 to 1. As shown in the last column of Table 2, dialysis of the HPLC purified enzyme against EGTA, followed by dialysis aginst water, removed a significant amount of bound calcium and zinc. Both EDTA and EGTA inhibit elastase. Under the conditions of our experiments, EGTA was a better inhibitor of elastase in thise experiment, and in some cases EDTA showed good inhibition ativity (not shown). Lineweaver-Burk and Schatchard replots of the primary kinetic data indicated EGTA was a noncompetitive inhibitor of elastase. The inhibition of HPLC purified elastase, dialysed against 0.05 M Tris-HCl, pH 7.3, by EGTA concentration is shown in Figure 6. 1.0 mM EGTA inhioted the elastase by over 50% and 10 mM EGTA inhitibed almost 75% of the original activity. It is important to point out the purity of

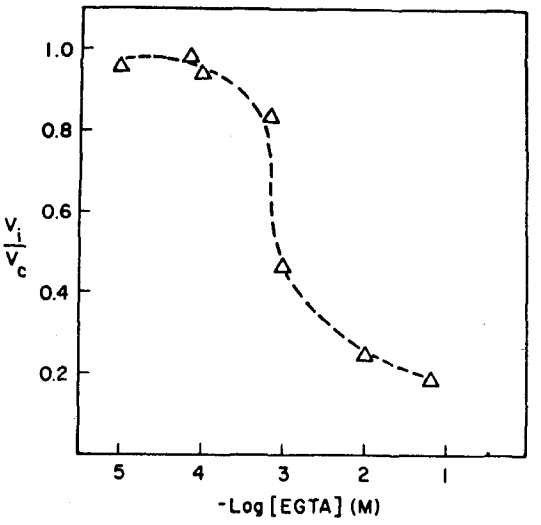

Fig. 6. Inhibition of elastase by EGTA : 3.4 ug of elastase was incubated in 0.2 ml of 50 mM Tris-HCl, pH 7.3 and with ^{3}H-elastin (12,000 cpm) at 37°C for 4 hours. Reactions were stopped by addition of 50 ul of unlabeled elastin suspended in 7% acetic acid and centrifuged for 5 minutes. 0.1 ml of the suppernatants were counted in 4 ml of schintilation fluid. Vc = activity of control. Vi = activity of EGTA inhibited samples.

elastase also influenced the amount of inhibition demonsrable. The curde preparations required higher concentrations of chelator to achieve equal inhibition; 50% inhibition of crude leukocyte elastase requires greater than 100 mM EGTA while, as shown above with highly prufied enzyme 1 mM EGTA is sufficient.

The preincubation time of EGTA with human leukocyte elastases was vaired from 5 minutes to 18

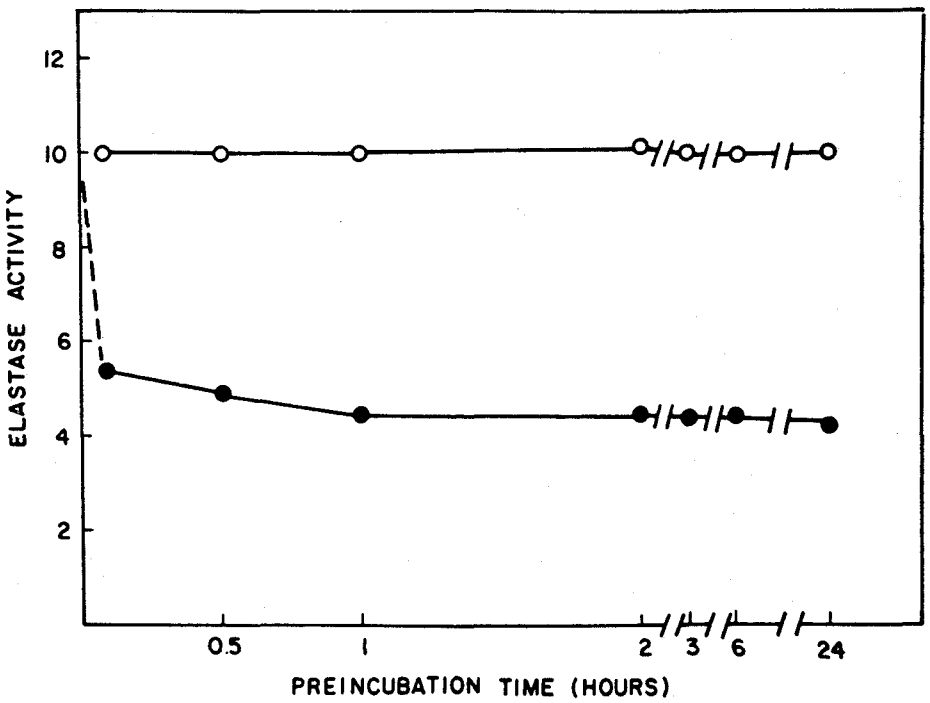

Fig. 7. Effect of preincubation time on EGTA inhibition : 2.6 ug of elastase was incubated with 36 mM EGTA in 250 ul of reaction medium containing 100 mM Tris-Cl, pH 7.3, at 33°C for various periods of time. Reactions were initiated by adding SANA to a final concentration of 4 mM. O ---- O : control ; ● ---- ● : EGTA effect.

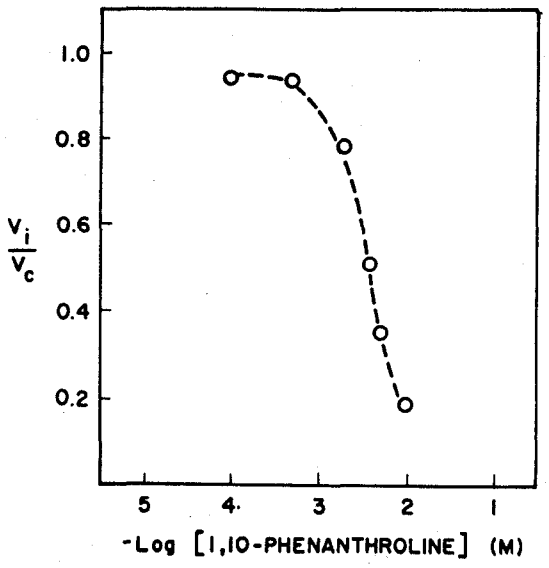

Fig. 8. Inhibition of elastase by 1, 10-phemanthroline: 4.2 ug of HPLC purified elastase was incubated with various concentrations of 1, 10-phenanthroline in 0.2 ml of reaction medium containing 100 mM NaCl and 50 mM Tris-Cl, pH 7.3, H at 37°C for 30 minutes. ^{3}H-elastin was then added to initiate the reaction. The reaction was stopped by adding unlabeled elastin suspended in 7% acetic acid. After centrifugation, 0.1 ml of supernatant was counted in schintilation fluid. Vc = activity of control, Vi= activity of inhibited sample.

hours. With the purest enzyme preparations, thirty minutes of preincubation resulted in almost 90% of the maximum inhibition obtainable. As shown in figure 7, most inhibition of human leukocyte elastase by EGTA took place in the first 5 minutes. Between 5 minutes and 60 minutes of preincuba-

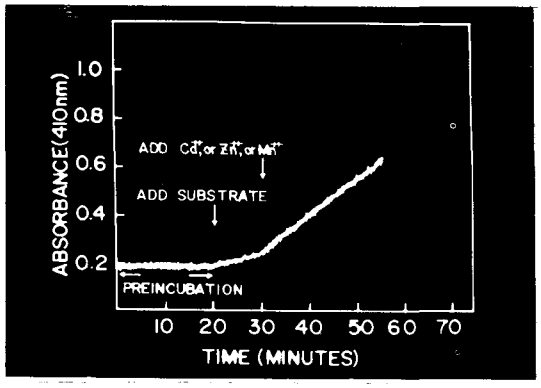

Fig. 9. Reversal of inhibition by Zn, Mn or Ca : 0.1 ug of elastrase was preincubated in 0.7 ml of reaction medium containing 50 mM EGTA, or 50 mM Tris buffer in control, for 20 minutes. 5 mM SANA was added, followed by the addition of the various cations indicated.

tion the rate of inhibition was slowed, reaching a plateau at 60 minutes.

Using various preparations of elastase inhibition experiments with 1,10-phenanthroline were performed either in the presence of 20% absolute ethanol (when the purity of the preparation was low and high concentrations of chelator were required) or in the presence of 50 mM Tris buffer, pH 7.3. Due to the efficacy of 1,10-phenanthroline optimal levels of inhibition could be attained with five minutes of preincubation time. The inhibition of HPLC purified elastase by 1,10-phenanthroline with the elastin substrate is depicted in figure 8. 50% inhibition was achieved at 5 mM concentration of this chelator.

Using SANA as substate, EGTA inhibition can be reversed by the addition of equimolar concentrations of $CaCl_2$, $ZnCl_2$ and $MnCl_2$ (fig. 9). The recovery of the elastase activity was 95% of control activity. However, the inhibition appears to be irreversible with more prolonged exposure to the chelators. If Mg^{++} or Na^{+} was substitued no recovery of activity over inhibited levels was seen.

DISOUSSION

The purification of large quantitises of elastase from human blood, employing one G-75 Sephadex and two HPLC elution protocols, yields highly purified enzyme with the added advantage of a relatively short production time. Regnier (1983) suggested that HPLC size exclusion columns should be operated at greater than 0.1 M-0.2 M ionic strength to overcome or minimize the electrostatic effects between suport materials and eluted proteins. At low ionic strength these columns have "exposed" negatively charged regions that can interact electrostatically with cationic proteins. Elastase, having isoelectric point of 9.5, are susceptible to electrostatic interactions with such columns, particularly if the eluant pH is in the acidic range. By carefully controlling the chromatographic conditions it was possible to take advantage of this electrostatic binding in order to separate elastase and catheptin G from the majority of proteins in extracts of leukocytes. Elastase and cathepsin G bound optimally to HPLC gel filtration columns of the TSK type in the presence of 10 mM sodium acetate and 5 mM NaCl at pH 5.5, and both elastase and cathepsin G were successfuly eluted without contaminating one another at different salt concentrations.

Elastase was successfully eluted in a relatively pure form(at least greater than 99% free of other proteins) and without a significant loss of activity (85 to 95% yield), using a salt gradient between 0.02 and 1.0 M NaCl at pH 5.5. The purified elastases showed specific characteristics of immunoprecipitation against human leukocyte elastastase and same substrate specificities against elastin and SANA.

The molecular weights of the three elastases isolated by HPLC were between 26,400 and 29,700 and these values were in agreement with Baugh & Travis (1976), Starkey & Barrett (1976b). The three bands of elastase have often been termed "isozymes". According to the current recommendations of the Commission on biological nomenclature of IUPAC-IUB (1977), isozymes are defined as multiple molecular forms of an enzyme occurring within a single species as a result of the presence of more than one structural gene. The definition of isoenzymes are multiple forms of enzymes which arise by the association of protein subunits that are themselves products of distinct structural genese (Moss, 1982). On the basis of this definition, and with regard to the chemical characterization reported (Ohlsson & Olsson, 1974; Baugh & Travis, 1976; Starkey, 1977), the "isozymic" nature of the various elastase forms has been prematurely conlcluded. Therefore, until the gene-

tic relationships between these various forms of elastase are clarified, they will be referred to as "forms" or species" of the elastase.

There seems to be complete agreement in the literature and our results that human leukocyte elastases are inhibited by Dip-F and Pms-F (Starket & Barrett, 1976b; Starkey, 1977). They were also inhibited by chelating agents; EDTA, EGTA and 1,10-phenanthroline which are known as inhibitiors of metalloprotease (Kang, 1985). From the results presented here neutrophil elastase should be considered as a metal binding proteinase or a metalloprotease in which the metal, likely Zn, can function in the regulation of catlytic activity. According to Vallee's criteria of the characteristics of metalloenzymes (1955), the following observations are used to support this conclusion; (1) the quantity of zinc increased at each step of purification, remaining constant when the enzyme was in its highest state of purity (Table 2), (2) a molar ratio of 2:1 between zinic and elastase was found at the final stage of purification, (3) removal of zinc resulted in the inhibition of the enzyme and, (4) the addition of zinc to the apoenzyme reversed the inhibition. Since the quantity of Ca in purified elastase was also notably high, and calcium can reverse EGTA inhibition, this element may be important for the activity of elastase.

The function of zinc in metalloenzynes falls into four categories: catalytic, structural, regulatory and noncatalytic. If zinc has a catalytic role then it is required and involved directly in catalysis and thus, upon removal in an inactive apoenzyme results. A regulatory Zinc plays a structural role when it is required for the stability of the protein (Vallee & Stein, 1959; Galdes & Vallee, 1983). Glutamate, histidine, tyrosine and cysteine, as well as serine hydrogen bonded to the water molecule, have been shown to participate in the coordination of Zn, e.g., in leucine aminopeptidase (Van Wart & Lin, 1981), alcohol dehydrogenase (Vallee & Coombs, 1959), carboxypeptidase A (Bradshaw *et al.,* 1969), carboxypeptidase B (Titani *et al.,* 1975; Schmid & Herriott, 1976; Vallee *et al.,* 1983).

The metal dependent character of elastases from many other sources has been reported, i.e., from Pseudomonase aerugionsa (Morihara *et al.,* 1965, Morihara and Tsuzuki; 1975), mouse macrophages (White *et al.,* 1980; Banda & Werb, 1981), human fibroblasts and smooth muscle cells (Goudeau, *et al.,* 1982; Schwartz *et al.,* 1984). Hormebeck demonstrated that elastase activity in human serum was inhibitied by 1,10-phenanthroline (Hornebeck *et al.,* 1983). As stated above, investigators, have also shown that the porcine pancreatic elastase can be inhibited by EDTA (Starkey & Barrett, 1976b). We have shown here that human leukocyte elastase can be inhibited by the chelating agents, EGTA and 1,10-phenanthroline, and that the inhibition may be reversed by Zn, Mn and Ca under defined conditions. Although neutrophil elastase has been classified as a serine protienase based on its susceptibility to Pms-F and insensitivity to low concentrations of EDTA, it appears that this enzyme may contain at least one, and possibly two, catalytic or structural zinc atoms and should be reconsidered as member of the metalloenzyme class.

Inhibition by chelating agents is not always due to the result of removal of a metal ion from the enzymes, but can be due to the binding of a chelator to the metal ion resulation in the induction of a conformational change in the enzyme. Thus, the inhibition of elastase by the various chelators could also be via this mechanism. The requirements for relatively high concentrations of chelators to reach maximum inhibition may be related to the high affinity constants of the elastase-Zn complex. If it is assumed that the affinity constant for elastase-Zn complexes is greater than 10^8, the value used as a guideline to distinguish metalloenzymes from metal-activated enzyme (Vallee, 1955; Galdes & Vallee, 1983), this value would be close to the affinity constant of the chelator-Zn complexes; i.e., the Kass of EGTA and EDTA for Zn at pH 7, is 10^8 and 10^{12}, respectively. Thus, equilibria competition for the metal would exist between the enzyme and the chelator. The reversal of the inhibition by calcium (figure 9) may be related to the release of Zn from a EGTA-Zn or elatase-Zn-EGTA complex when the calcium is added to the reaction mixture. The restoration of activity by Mn may be due to a similar displacement of Zn, or alternatively that Mn, itself a transition metal, replaced a catalytic (or structural) Zn. Na and Mg, which are not transition metal which are required to be metal ion in metalloenzyme according to Vallee's criteria, had no effect on the reversal of EGTA inhibition. It may also related to the stability constants for both Na-and Mg-EGTA which are much lower than that of Zn- and Mn-EGTA complexes. Our findings showed that elastase activity was irreversibly lost if the inhibition time was extended beyond one hour. Therefore, dialysis alone could not be used to remove EGTA-Zn complexes from the inhibited apoen-

zyme. This may also indicate that the Zn atoms may provide a degree of structural stability to elastase. These characteristics proves indirectly that this enzyme is Zn-metalloenzyme.

The reason that the inhibition of leukocyte elastase has not been shown by other investigator may be due to a number of factors. The first is related to the purity of the elastase preparations. Small amounts of a protein contaminant may have been bound to the elastase near its metal binding site which impaired access of the chelator. Alternatively, protein impurities containing bound "adventitious" metals may have competed with the chelator for essential metals in the molecules of elastase. In some case when less pure elastase preparations were tested for chelator sensitivity higher concentrations of chelator and longer preincubation were required to demonstrate inhibition. Second, EDTA is much weaker inhibitor than EGTA in our experiments and is unpredictably effective, especially if reatively crude preparations of enzyme are tested. Third, inhibition by EDTA, EGTA and 1,10-phenanthrolin is poor in high salt conditions and where enzyme activity is accelerated. An cceleration of activity can occationally be detected when EDTA or EGTA is used as inhibitor at high ionic strenght in certain experimental design, probably due to the increase in ionic strength the reaction mixture contributed by the buffer used to neutralize the effect of chelator. The "causal" buffering of chelators may result in the ionic strength stimulation of the enzyme rather than inhibition.

Inhibition of human leukocyte elastase by chelating agents showed noncompetitive inibition characteristics.

Generally on a kinetic basis the testing of noncompetitive inhibitors should be determined at substrate concentrations at or below the Km for that substate (Cornish-Bowden, 1979). Rarely has this been taken into consideration when elastase has been assayed for sensitivity to EDTA. Another important factor to see the inhibition properties of human leukocyte elastase by chelating agents is optimum concentration of salt in the reaction medium, in our laboratory, salt concentration was kept at the level to have 60 to 80% of control activity.

Pancreatic elastase has been classified as a serine proteinase on the basis of its inhibition by Pms-F and the apparent lack of effect of metal ion chelators (Hartley, 1960), although, as has been pointed out pancreatic elastase can be inhibited by EDTA (Lamy, Craig. & Tauber, 1961; Starkey, 1977). Further, Pms-F can react with the hydroxyl radical of threnoine and tyrosine and the SH group of cysteine; each of these amino acids has been shown to coordinate Zn or water molecule bound Zn in metalloenzymes. Serine is known to be hydrogen bonded to the water molecule associated with a catalytic Zn in alkaline phosphatase and lies in the active site pocket of numerous other metalloenzyms. We are not sure exact position of the Zn in three dimensional structure of human leukocyte elastase, however, our data suggest that the neutrophil elastase should be reconsidered for reclassification as a metalloenzyme, although serine residues may play a role in maintaining its catalytic ability.

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＝국문초록＝

사람의 백혈구 내에 있는 Elestase : 순수부리, 금속이온의 화학량, 그리고 Chelating 효과에 의한 활성도 조절

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사람의 혈액으로부터 Elastase를 분리하는 새로운 방법을 개발하여 순도 높은 효소를 얻고 이 효소에 의하여 발생되어지는 질병의 예방과 치료에 응용하기 위하여 이 효소의 근본적인 성질 규명을 시도하였다. 정제 방법으로는 Sephodex G-75를 이용한 1회의 액체 크로마토그라피와 HPLC을 이용한 2회의 코마토그라피를 거치는 2단계 방법을 사용하였다. 이때 얻어진 효소는 분자량이 26,000~29,700 사이에 있는 3개의 다른 분자량을 가진 물질로 확인되었으며, 항체 반응에서 사람의 결구내 Elastase의 특성을 나타내었다. 함유하고 있는 미량의 금속이온을 분석한 결과 정제하는 단계에 따라 Zn 이온의 효소분자에 대한비는 증가하였으며 가장 순수한 분자내의 효소분자와 Zn 이온과의 비는 1 : 2였다. 이 효소는 chelating agents에 의하여 활성도를 잃게 되었으며, 반대로 chelating agents에 의하여 활성도를 잃고 있는 반응 medium에 그 chelaing agents의 농도를 초과하는 2가 이온 즉 Zn 이온, Ca 이온, 그리고 Mn 이온을 넣으면 그 활성도는 원상복귀되나 Mg이온에 의하여는 그 활성도가 원상회복 되지 않았다.

이 모든 성질을 종합하면 사람의 Neutrophil granule에 있는 Elastase는 지금까지 알려진 바와 같이 Serine protease임과 동시에 metalloenzyme으로 제고되어야 한다고 제안한다. 나아가서 chelating agents에 의하여 이 효소의 활성도를 조정할 수 있다는 것은 이 효소에 의하여 일어나는 질병의 치료에 응용할 수 있는 가능성도 보여준다.