

# RAPID PURIFICATION AND MOLECULAR CHARACTERIZATION OF HUMAN NEUTROPHIL ELASTASE

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## = 국문초록 =

Elastase에 의해 야기되는 질병의 예방 및 치료 응용을 위하여는, 먼저 이 효소가 고순도로 분리되고 그 근본적인 성질의 해명이 이루어져야 한다. 고순도의 elastase를 얻기위해, 사람의 전혈로부터 Hypaque-Ficoll step gradient에 의해 순수한 neutrophil을 얻은 다음 이것을 extract로 만들어 액체크로마토그래피를 실시하였다. Ultrogel AcA54 gel filtration을 거친 후 CM-sephadex 이온 교환 크로마토그래피를 적용하였는데, 이때 유출 완충액 속에 0.1% Brij 35를 첨가하여 elastase의 순도를 더욱 높일 수 있었다.

이 정제된 elastase는 SDS-PAGE를 통해 MW 28,000, 29,000, 30,000의 서로 다른 3개의 밴드로 확인되었으며, 그간 수많은 연구자들간에 elastase의 분자량에 대해 심한 견해 차이를 보여온 것은 그 분자적 성질이나 분자량이 유사한 cathepsin G가 혼재하거나 elastase분자내 혹은 분자간 S-S결합의 차이에 의함을 알 수 있었다.

## INTRODUCTION

The source degrading insoluble elastin is believed to be from phagocytic cells, in particular neutrophils<sup>1,2</sup> and macrophages<sup>3</sup>. Human neutrophils have three proteinases-a specific collagenase<sup>4</sup>, an elastase<sup>5,6</sup> and a chymotrypsin-like proteinase cathepsin G<sup>7</sup> which are capable of digesting a wide variety of tissue components, such as native collagen<sup>8</sup>, lung and arterial elastin<sup>9</sup>, cartilage proteoglycan<sup>10</sup>, vasacular basement membrane<sup>11</sup>, and bacterial cell wall peptidoglycan<sup>12</sup>. In 1968, Janoff and Scherer

were the first to report on the presence of elastase activity in granules of human neutrophils<sup>19</sup>. Human neutrophil elastase (HNE, EC 3, 4, 21, 11) that is located in its azurophil granules<sup>61</sup> can destruct the extracellular matrix, and thus it might result in acute inflammation<sup>10151</sup>, rheumatoid arthritis<sup>111</sup>, tissue injury<sup>161</sup>, and lung emphysema<sup>21</sup>. To investigate the role of HNE in these diseases, it might have been preceded the security of highly pure elastase and its authentic characterization. Extensive researches presented that HNE has maximum activity at neutral pH<sup>31</sup> and has been categorized as serine endopeptidase<sup>341</sup> and metalloenzyme<sup>221</sup>. And, it has been reported the crystallization and X-ray diffraction analysis of crystals of a HNE<sup>4391</sup> and its primary structure<sup>331</sup>. Recently, nucleotide sequence of HNE gene was elucidated<sup>81</sup>. But, the enzymatic properties of HNE still has been obscure and its molecular weights has been disagreeing for a long time. Therefore, highly pure purification of HNE should be of great value for its definite characterization.

This report describes the excellent purification process and the accurate determination of molecular weight of HNE.

## MATERIALS AND METHODS

### Materials

Hypaque(sodium diatrizoate), Ficoll(type 400, MW 400,000), N-Suc-(Ala)3-PNA (SANA), N-Benzoyl-DL-Phenyl-Alanine- $\beta$ -Naphthylamide(BPNE), Fast Garnet GBC Base, and Brij 35 were purchased from Sigma. Ultrogel AcA54, CM-sephadex C-25, and electrophoretic chemicals were purchased

from LKB, Pharmacia, and Bio-rad respectively. Other chemicals were used G.R grade.

Anti-human neutrophil elastase (anti-HNE) was a generous gift of John, S.Mort, Ph.D, joint diseases laboratory, Shriners Hospital for Crippled Children (Quebec), Canada.

### Isolation of Human Neutrophil

Human neutrophils were isolated from peripheral blood of normal donors as described previously<sup>241</sup>. Whole blood was carefully layered on the 3-step gradient of Hypaque-Ficoll solution (density; 1,080, 1,122, 1,133g/ml) and centrifuged at 300 g for 25 minutes at room temperature.

### Purification of Human Neutrophil Elastase

Pure neutrophils were homogenized and centrifuged. The supernatant was then chromatographed by two steps with Ultrogel AcA54 and CM-sephadex C-25.

#### A) Gel Filtration

Gel filtration was performed on Ultrogel AcA54. The procedure was same as previous method<sup>211</sup>. Every other fraction was assayed for protein concentration<sup>51</sup>, haemoglobin, elastase<sup>23361</sup>, and cathepsin G<sup>341</sup>. The fractions demonstrating elastase activity were pooled, concentrated, and dialysed for further purification.

#### B), Ion-Exchange Chromatography

The CM-sephadex column was equilibrated with 0.01M sodium acetate, pH5.5, containing 0.15M NaCl and 0.1% Brij 35 at 4°C and the elastase fraction from Ultrogel AcA54 was dialysed against the same buffer before it was applied to the column. Elution was carried out by a linear gradient of sodium chloride from 0.15M to 1.0M, in 0.05M sodium ace-

tate pH5.5 Every other fraction was assayed for elastase and cathepsin G. The fractions of peak elastase activity, which were free of catheptic activity, were pooled and concentrated to 1/10 of the original volume by ultrafiltration.

### Enzymatic Assay

#### A) Elastase Assay with SANA

The esterolytic activity of elastase was determined spectrophotometrically at 410nm by monitoring p-nitroaniline released from the synthetic substrate, SANA<sup>25,361</sup>.

#### B) Cathepsin G Assay

The standard incubation mixture contained an aliquot of column fractions, 100mM Tris-Cl pH7.3, 0.1% Brij 35 and 4mM BPNE (freshly dissolved in dimethylsulfoxide). The reaction was carried out at 37°C for 90 minutes and stopped by the addition of coupling reagent. The coupling reagent was prepared by modification of Starkey et al.<sup>23,340</sup> The reaction mixture was incubated at room temperature for 5 minutes until the color was developed, then followed by centrifugation. The absorbance was read at 520nm.

### Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a modification of the method of Laemmli.<sup>25</sup> The gel concentration was 10% and samples were prepared by acetone-methanol precipitation before it was applied to the well. After running, protein bands were stained with 0.1% Coomassie brilliant blue R.

### Ouchterlony Immunodiffusion Assay

Double immunodiffusion was performed in agar by the method of Ouchterlony.<sup>29</sup> Sam-

ples from the various purification stages were tested for their reactivity with anti-HNE, which was raised in a sheep. Diffusion was processed for 24 hours at 37°C in a temperature and humidity controlled chamber. The gel plate was stained in 1% amido black and destained in 2% acetic acid and 12% glycerol.

## RESULTS

### Purification of Human Neutrophil Elastase

After centrifugation at 300g for 25 minutes at room temperature, 3 different cell fractions were observed. The third band was predominantly neutrophils and the cell purity was 96.2%<sup>24</sup>. Approximately  $4.8 \times 10^6$  cells could be routinely obtained from 100ml whole blood by this procedure.

The gel filtration of Ultrogel AcA54 was a crucial step for removal of high molecular weight contamination. This gel had better resolution for elastase and cathepsin G than sephadex G-75. The specific activity of the elastase eluted from Ultrogel AcA54 was 877.2 at our specific reaction condition (Table 1) and the  $K_m$  for elastase with SANA was 14mM. But, as previously mentioned<sup>21</sup>, the elastase fractions always contained small amounts of cathepsin G.

For separation of HNE from the trace amounts of cathepsin G, CM-sephadex ion-exchange chromatography was essential. Better resolution of HNE was obtained using 0.1% Brij 35 than no Brij 35 under the same conditions (Fig. 1). Using 0.1% Brij 35 was an important factor for preparing both pure elastase and cathepsin G. The elastase fractions were free of cathepsin G and the cathepsin G fractions also were free of elastase. As seen in

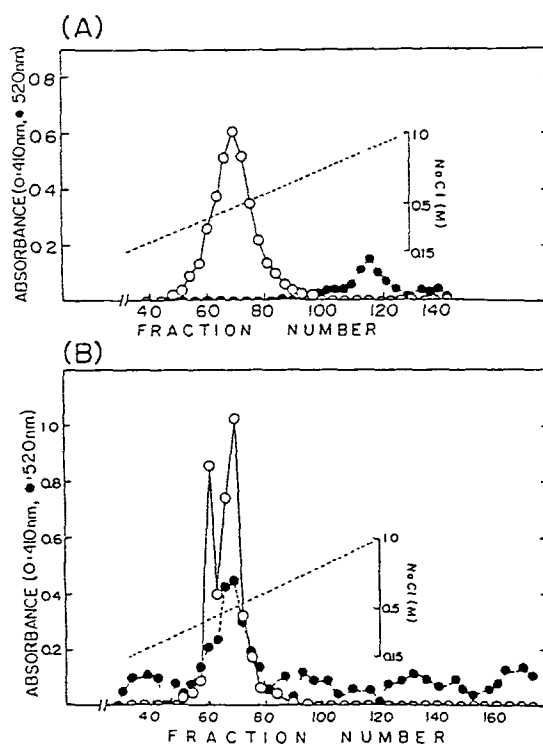
**Table 1.** Purification of Human Neutrophil Elastase

Step	Total protein (mg)	Specific activity ( $\mu\text{M}/\text{mg} \cdot \text{min}$ )	Total activity ( $\mu\text{M}/\text{min}$ )	Recovery (%)	Fold purity
Crude extract	16.4	88.2	1447.1	100	1.0
Ultrogel AcA54	1.0	877.2	877.2	60.6	9.9
CM-sephadex C-25	0.14	4746.8	664.6	45.9	53.8

Table 1, the specific activity of the elastase from this step was 4746.8 at out specific reaction condition. This represents an 53.8-fold purity and a recovery of 45.9%. The immunological relationship of the HNE antisera to the enzymes at the various purification stages is shown in Fig 2. In this immunodiffusion test, 4 samples containing elastase activity formed precipitin band. No cross-reactivity with cathepsin G from Ultrogel AcA54 and CM-sephadex was detected. As will be described subsequently, the elastase obtained by CM-sephadex was sufficiently pure for further basic studies.

### Molecular Weight

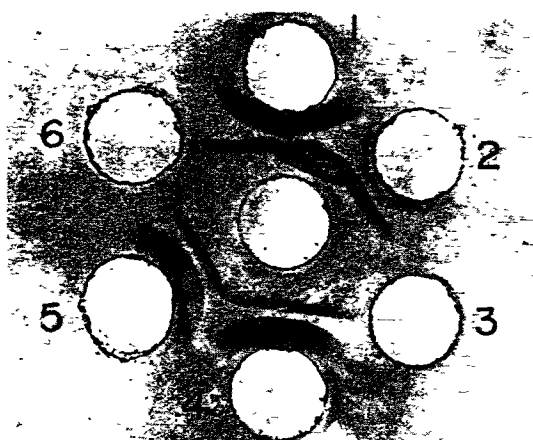
The purity of the enzymes was examined by polyacrylamide gel electrophoresis in the presence of SDS. The purity of samples at the various purification step was demonstrated in Fig 3. Elastase or cathepsin G pool from Ultrogel AcA54 and unbound proteins from CM-sephadex were mixed with many other proteins (Fig 3, lanes 2 and 3). CM-sephadex purified elastase was always free of cathepsin G and not contaminated with other proteins. The elastases showed the variety of their molecular weights: 1) 28,000, 29,000 and 30,000 (Fig 3, lanes 6 and 7); 2) 31,000, 32,000, and 33,000 (Fig 3, lanes 8 and 9), and 3) mixed state of 1) and 2) (Fig 4, lane 2). These various patterns substantiate the fact



**Fig. 1.** Ion-Exchange Chromatography of Human Neutrophil Elastase on CM-sephadex C-25

The column (15 x 30 cm) was equilibrated with 0.05 M sodium acetate, pH 5.5, containing 0.15 M NaCl and (A) 0.1% Brj 35 (B) no Brj 35, and eluted with a linear gradient to 1.0 M NaCl (○---○) elastase (410 nm), (●---●) cathepsin G (520 nm).

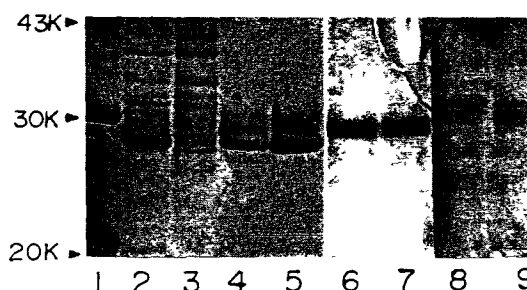
that the molecular weights of the elastase are very flexible because of structural changes during processes of the purification and sample preparation. For investigating the reasons of band variability, we selected 2-



**Fig. 2.** Double Immunodiffusion between Anti-HNE and the Protein Samples at Various Purification Stages

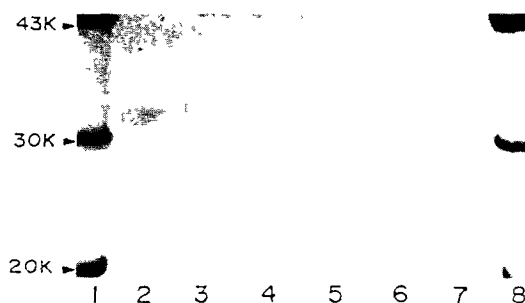
Antibody to HNE was placed in the center well. 1. CM-sephadex purified elastase(FN 50-65 of Fig 1-(A)) 2. CM-sephadex purified elastase(FN 66-90 of Fig 1-(A)) 3. CM-sephadex purified cathepsin G(FN 115-130 of Fig 1-(A)) 4. CM-sephadex purified elastase and cathepsin G(FN 55-80 of Fig 1-(B)) 5. Ultrogel AcA54 purified elastase 6. Ultrogel AcA54 purified cathepsin G

mercaptoethanol reducing agent of disulfide bonds. Because the elastase molecules have rich S-S bonds 10% and 20% of 2-mercaptoethanol treated samples showed significantly different patterns each other(Fig 4 lanes 2 and 3, 6 and 7). 20% of 2-mercaptoethanol treatment changed elastase molecules below 30 K into above 30 K. In case of pure cathepsin G migration of the molecules from low molecular to high molecular weight was negligible(Fig 4 lanes 4 and 5). When elastase was mixed with cathepsin G, the band pattern was much confused(Fig 3 lanes 4 and 5). Probably the trace amounts of cathepsin G effect changes of the electrophoretic mobility of elastase. As



**Fig. 3.** SDS-Polyacrylamide Gel Electrophoresis of Samples from Each Purification Step

Lane 1. globular protein standards included ovalbumin(MW43,000), carbonic anhydrase-(MW30,000), and soybean trypsin inhibitor(MW20,100). lane 2. cathepsin G from Ultrogel AcA54, lane 3. unbound proteins from CM-sephadex, lanes 4 and 5. elastase and cathepsin G from CM-sephadex, lanes 6-9 elastases from CM-sephadex



**Fig. 4.** SDS-Polyacrylamide Gel Electrophoresis of Samples from CM-sephadex

2-Mercaptoethanol concentration of sample buffer was varied and others are same conditions. Lanes 1&8 globular protein standards, lane 2. elastase(10% 2-mercaptoethanol), lane 3. elastase(20% 2-mercaptoethanol), lane 4. cathepsin G(10% 2-mercaptoethanol), lane 5. cathepsin G(20% 2-mercaptoethanol), lane 6. elastase and cathepsin G(10% 2-mercaptoethanol), lane 7. elastase and cathepsin G(20% 2-mercaptoethanol)

cathepsin G was absolutely removed from the mixture of elastase and cathepsin G, pure elastase returned to the regular patterns on SDS-PAGE. Consequently, the important facts to elucidate elastase characteristics are its purity and preparation system.

## DISCUSSION

It is an important prior step for purification of HNE to isolate pure neutrophil from normal donors in a brief time. The next important step of purification of HNE is molecular level of purification from the pure neutrophils. Although gel filtration, ion-exchange chromatography and affinity chromatography are conventionally used for purification of HNE, their combination procedures are various in different laboratories. At each step, the selection of column matrix are also specific. It is very difficult to compare the results of one laboratory with other's directly since experimental conditions in different laboratories are different. However, in general, purification of HNE by 2-step procedure showed higher yield than 3-step procedure. Highly pure HNE was recovered 64% and 32% of original weight, through a 3-step procedure involving affinity chromatography on Aprotinin-sepharose, ion-exchange chromatography on CM-cellulose, and gel filtration chromatography on AcA44<sup>12</sup> and through a 2-step procedure involving affinity chromatography on Sepharose-Trasylol and ion-exchange chromatography on CM-cellulose<sup>1</sup>, respectively. Pure HNE was recovered 45.9% through a 2-step procedure, i.e., gel filtration on Ultrogel AcA54 and ion-exchange chromatography on CM-sephadex C-25 (Table 1) in our laboratory. If the purity of final product is similar

it will be very reasonable to simplify chromatography procedures.

Purification of HNE required special caution to control because of its endopeptidase property, pH, buffer system, temperature, and detergent. Addition of Brij 35 to buffer system was critically important for purification of HNE with Ultrogel AcA54 and CM-sephadex C-25. Purity of the samples purified with or without Brij 35 in ion-exchange chromatography and gel filtration was comparable (Fig 1). Starkey & Barrett primarily also showed good resolution by using Brij 35 for extraction of neutral proteinases from human spleen tissue<sup>35</sup>. In the immunodiffusion precipitation against anti-HNE antibody, there was a striking contrast between the purified elastase and cathepsin G (Fig 2, well 3 and 6).

There had been a wide difference in molecular weight of HNE for a long time, and it was one of the most important argument for its fundamental characterization. Molecular weights of HNE which have been reported were between 22,000 and 36,500 with a remarkable contrast, 34,400<sup>9</sup> or 33,000, 34,000, and 36,500<sup>28</sup>; 27,000<sup>32</sup>, 28,900<sup>11</sup>, or 22,000<sup>37</sup>. Generally speaking, the formers are above 30,000 and the latters are below 30,000. On the contrary, some colleagues reported the molecular weight as 27,500, 28,000 and 31,000<sup>12</sup>. Having an extremely careful experiment and observation of these diverse phenomena (Fig 3, lanes 6 and 7, 8 and 9) could be reached us to classify the molecular weight into two groups, i.e., above 30,000 and below 30,000, but mostly mixed state of the two categories. Up to now, the reasons of appearing of two groups of different molecular weight have been ex-

plained as autolytic cleavage of HNE molecule or disparity of carbohydrate side chain. We could demonstrate the possibility of variation of the molecular weight of HNE by protein denaturing condition or renaturation (Fig 4). It has been known that HNE molecules form rich inter- or intra-disulfide bonds. When we added high concentration of 2-mercaptoethanol to the sample buffer, remarkable change of molecular weight of HNE could be seen, i.e. mixed state of molecules of above 30,000 (31,000, 32,000 and 33,000) and below 30,000 (28,000, 29,000 and 30,000) could be returned to molecular weight above 30,000 by treatment of 20% of 2-mercaptoethanol (Fig 4, lanes 2 and 3). Cathepsin G was not greatly influenced by 2-mercaptoethanol during SDS-PAGE (Fig 4, lanes 4 and 5). Most investigators including us agree the molecular weights of human neutrophil cathepsin G are between 28,000 and 31,000<sup>2,6</sup>. Owing to the similarity of molecular weight of both cathepsin G and elastase located in same azurophilic granules of neutrophil together and highly positive charged molecular characteristics, many investigators had been faced with technical difficulty for purification of HNE. When elastase molecule was mixed with cathepsin G, the band mobility on SDS-PAGE was much complex (Fig 3, lanes 4 and 5). This complexity may be caused by not only simple mingling of two molecules but also complex inter-influence between two different groups of enzymes. Conclusively, we suggest that the discrepancy or the diversity of molecular weights of HNE was caused by mainly the disparity of intra-molecular disulfide bonds of HNE.

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