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Novel method of purification of human leukocytic elastase using adsorption on a high-performance liquid chromatography gel permeation column

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ABSTRACT

Neutrophil elastases are serine proteinases released during acute and chronic inflammatory states. We have developed a novel isolation method for neutrophil elastase, involving conventional gel chromatography followed by adsorption of protein at low ionic strength on a high-performance liquid chromatography gel permeation column. The bound elastase is then eluted by application of higher ionic strength. This adsorption step at low ionic strength, a step to be avoided in most purification methods, was used to advantage here to allow isolation of homogeneous material. This purification procedure should be useful for quick, simple bulk preparation of the enzyme.

INTRODUCTION

Purification schemes that are fast and efficient have been published for human leukocytic elastase [1,2]. While using one of these methods [2] we modified the procedure to take advantage of the speed and resolution of high-performance gel permeation rather than conventional gel permeation chromatography. Upon attempting to define the optimal ionic strength for the buffer we found that the ionic strength could be varied to allow adsorption and desorption of elastase from the matrix. We report here a modified elastase isolation procedure which takes advantage of the speed of high-performance gel permeation, the cationic charge of elastase and the anionic nature of the gel permeation matrix.

EXPERIMENTAL

Chemicals and reagents

Hypaque-Ficoll, elastin, enzyme substrates and conventional chromatography resins were obtained from Sigma (St. Louis, MO., U.S.A.). Gel electrophoresis reagents were from Bio-Rad Labs. (Richmond, CA, U.S.A.).

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Preparation of crude human leukocyte extract

Crude human leukocyte extract was prepared with similar results, using either one of the following protocols. In the first method, 450 ml of whole blood from normal volunteer donors were treated with 63 ml of 0.1 *M* citrate–0.1 *M* phosphate–dextrose. The mixture was centrifuged at 4000 *g* for 10 min at 4°C. The buffy coat was then recentrifuged at 12 000 *g* for 30 min, and then leukocyte-enriched layer, located above a small layer of red blood cells, was removed and washed several times in water to lyse remaining adherent erythrocytes. The washed leukocytes, which were predominantly polymorphonuclear leukocytes, were then mixed with an equal volume of 0.5 *M* Tris–HCl, pH 7.3, containing 2 *M* NaCl and 0.05 *M* $CaCl_2$ and homogenized in a Model STD Tissumizer (Tekmar) by 5-min bursts at maximum speed. Soluble material was recovered by centrifugation at 35 000 *g* for 30 min and used for further purification. In a second extraction protocol with fresh blood, polymorphonuclear leukocytes were first separated from other leukocytes by Hypaque-Ficoll density gradient centrifugation [3] and adherent red cells removed by hypotonic hemolysis. Leukocytes were homogenized as above.

Protease assays

Elastase activity was measured by hydrolysis of a synthetic substrate, SANA (succinyl-Ala-Ala-Ala-*p*-nitroanilide), and by hydrolysis of elastin. Both assays were used in parallel and gave similar results when elastase derivatives were compared. The SANA assay, a modification of the method described by Starkey and Barrett [4], was performed by addition of elastase preparations (0.03–0.3 μg) in 50 m*M* Tris–HCl, pH 7.3, containing 150 m*M* NaCl and 5 m*M* $CaCl_2$ to SANA in buffer in a final volume of 200 μl. The reaction was conducted at 37°C for 10–20 min in microtiter wells or cuvettes and hydrolysis monitored in a Titertek multiscan spectrophotometer at 410 nm or by direct readings in a thermostated Gilford/Beckman spectrophotometer. The specific activity was defined as μmol SANA hydrolyzed per mg protein per h. In order to assay elastinolytic activity toward elastin, radiolabeled elastin was prepared by chemical reduction, with [^{3}H]sodium borohydride, of desmosine and isodesmosine cross-links of bovine nuchal ligament elastin [5]. The specific activity of radioactive elastin was 1000–1500 cpm/μg. To assay, elastase (0.3 μg) was mixed with labeled elastin (25 000–100 000 cpm) in 250 μl of assay buffer at 37°C with continuous agitation. The reaction was stopped by addition of 100 μl of 0.5 *M* acetic acid, the mixture centrifuged at 10 000 *g* for 10 min, and the radioactivity in the supernatant measured. Cathepsin G activity was assayed using benzoyl-*dl*-phenylalanine naphthyl ester as substrate [6]. Collagenase activity toward type I collagen was determined by mixing constant amounts of test sample and collagen and after various times subjecting the reduced mixtures to sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) [7] and observing the appearance of the 75-kD alpha$_1$ and alpha$_2$ reaction products. Type I collagen was isolated from rat tail tendons as described [8].

Gel permeation chromatography

Conventional gel permeation chromatography was performed on a Sephadex G-75 column (100 cm × 2.5 cm I.D.) equilibrated with a solution of 200 m*M* NaCl, 5 m*M* $CaCl_2$, 0.1% Brij-35 and 50 m*M* Tris HCl, pH 7.3. All fractions were assayed for protein [9], cathepsin G activity, collagenase activity and elastase activity toward SANA and elastin.

High-performance gel permeation chromatography

High-performance liquid chromatography (HPLC) gel permeation was performed on a BioSil TSK 250 column (Bio-Rad Labs.) which was equilibrated with 20 m*M* NaCl, 10 m*M* sodium acetate buffer, pH 5.5, containing 0.1% Brij-35 and developed at 1.0 ml/min at 22°C. Elution was monitored at 280 nm. Samples of 8–16 ml were applied by repetitive injections of 2 ml every 10 min. After the absorbance reached baseline levels, a linear gradient of equilibrating buffer and equilibrating buffer adjusted to 1 *M* NaCl was applied. In a second HPLC gel permeation step, concentrated samples from this column were re-applied to this same column after re-equilibration in 1 *M* NaCl, 0.05 *M* Tris–HCl, pH 7.3. Use of a TSK 3000 column in this step gave similar results.

Immunologic identification of elastase

Immunologic identification of elastase was performed by Ouchterlony diffusion using antibodies to human neutrophil elastase kindly provided by Dr. Carlo Mainardi (Rutgers Medical School) and Dr. Sigfried Neumann (E. Merck, Darmstadt, Germany). Diffusion was allowed to proceed for 18 h at 37°C.

RESULTS

Purification of neutrophil elastase using a novel method

Aliquots of the crude leukocyte extract were chromatographed at 4°C on a Sephadex G-75 column (Fig. 1). All samples were assayed for elastase, collagenase and cathepsin G activities. Elastase eluted in a broad peak, separated from the majority of the proteins. Some of the collagenase and cathepsin G overlapped partially with the elastase peak in some of the chromatograms. However, the addition of 0.1% Brij 35 to the buffer resulted in better separation of these enzymes. Fractions containing elastase activity with some cathepsin but no collagenolytic activity were pooled, concentrated ten-fold by ultracentrifugation through an Amicon PM-10 membrane and subjected to HPLC gel permeation in lower ionic strength, 20 m*M* NaCl, 10 m*M* sodium acetate, pH 5.2 (Fig. 2). Arrow 1 indicates the start of repeated injections of the pool of concentrated crude elastase activity from the previous Sephadex G-75 gel permeation column. Up to 6–16 ml, more than 60 times the recommended maximum volume for this size column, were loaded onto the TSK 250 SW column. When the baseline had returned to the absorbance of the buffer, the full scale absorption was changed to

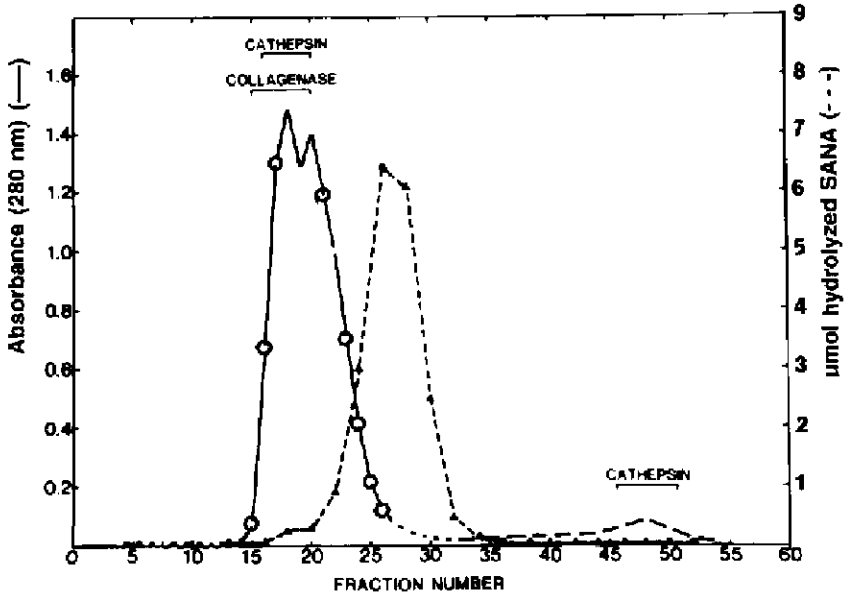

Fig. 1. Chromatography of human leukocyte extract on Sephadex G-75. The column (100 cm × 2.5 cm I.D.) was equilibrated with 50 m*M* Tris HCl, pH 7.3, containing 200 m*M* NaCl and 5 m*M* $CaCl_2$ at 22°C. Leukocyte extract (5 ml) was loaded and eluted at a flow-rate of 24 ml/h. (--○--) Protein; (--▲--) elastase activity against SANA. Areas that contained collagenase and cathepsin G activity are shown.

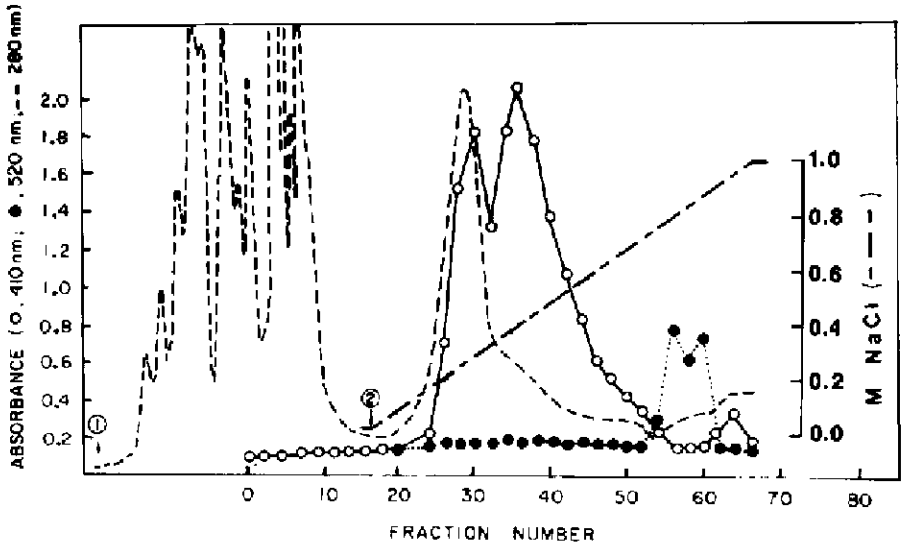

Fig. 2. Purification of elastase by adsorption of the protein on the HPLC column. The TSK 250 column was equilibrated with 10 m*M* sodium acetate (pH 5.5) containing 20 m*M* NaCl and 0.1% Brij 35 at a flow-rate of 1.0 ml/min at 22°C. Concentrated Sephadex G-75-derived elastase material (10 ml) was loaded, by repeated injections of 2.0-ml samples, beginning at arrow 1. After the absorbance returned to baseline, a linear NaCl gradient (0.02–1 *M*) was started (arrow 2) as shown in ordinate to right (- - -). (——) Protein absorbance at 280 nm; (--○--) elastase activity using SANA at 410 nm; (--●--) cathepsin G activity at 520 nm. Note that the elastase activity is separated from other activities and that a protein peak that correlates with elastase activity is apparent. Fractions 27–45 were combined for further purification.

0.08 and the salt gradient was initiated (arrow 2). Elastase activity eluted in two main peaks between 0.4 and 0.6 *M* NaCl. After its desorption from the HPLC column, this HPLC-purified elastase, which eluted in fractions 27–45, was then concentrated ten- to to twenty-fold and reapplied to the TSK 250 SW column for further purification. Because of the high ionic strength employed (1.0 *M* NaCl in 10 m*M* sodium acetate buffer), binding of proteins to the column was prevented, and separation was achieved by the molecular sieving property of the column (Fig. 3). Assays of each of the column fractions with SANA and [^{3}H]elastin demonstrated a single symmetrical peak of elastase activity.

The purification recoveries at various stages are shown in Table I; total purification fold was 555 with a yield of 33%. The isolated and reduced elastase showed bands of 31, 32 and 33 kDa on SDS-PAGE (Fig. 4) similar to the molecular mass reported by others [1,4].

In Ouchterlony diffusion assays, the isolated material and less purified fractions reacted with antibodies to neutrophil elastase but not with antibodies to cathepsin G (data not shown).

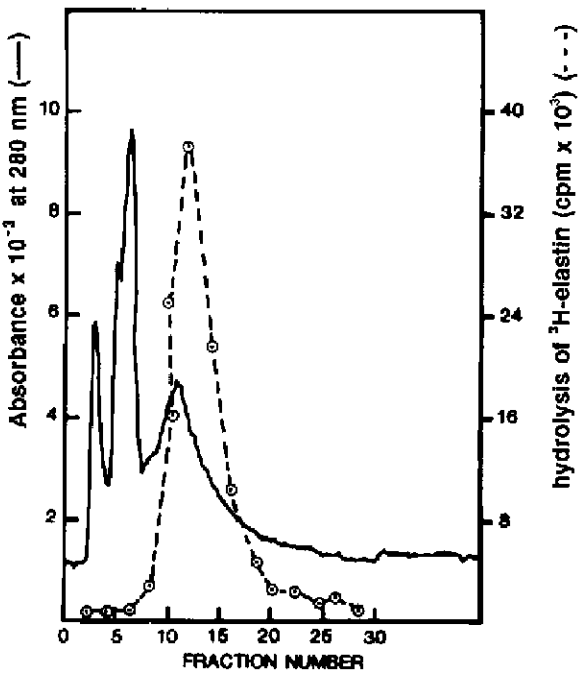

Fig. 3. Further purification of elastase by HPLC. The TSK 250 SW column was equilibrated with 50 m*M* Tris HCl, pH 7.3, containing 1.0 *M* NaCl. Concentrated elastase from the previous purification was dialyzed against the 50 m*M* Tris–HCl, pH 7.3 buffer containing 1.0 *M* NaCl and eluted at a flow-rate of 1.0 ml/min at 23°C. The column was monitored continuously at a wavelength of 280 nm (——). (--○--) Elastase activity against radiolabeled elastin. Assays using SANA as the substrate gave identical results.

TABLE I

PURIFICATION CHART

Step	Total protein (mg)	Specific activity[a] (μmol/mg/h)	Total activity[a] (μmol/h)	Yield (%)	Fold purity
Crude	27.2	0.53	14	100	1
G-75	3.6	3.78	14	94	7.1
HPLC 1st	0.085	63.5	5.4	38	120
HPLC 2nd	0.016	294	4.7	33	555

[a] Activity based on μmol hydrolyzed SANA.

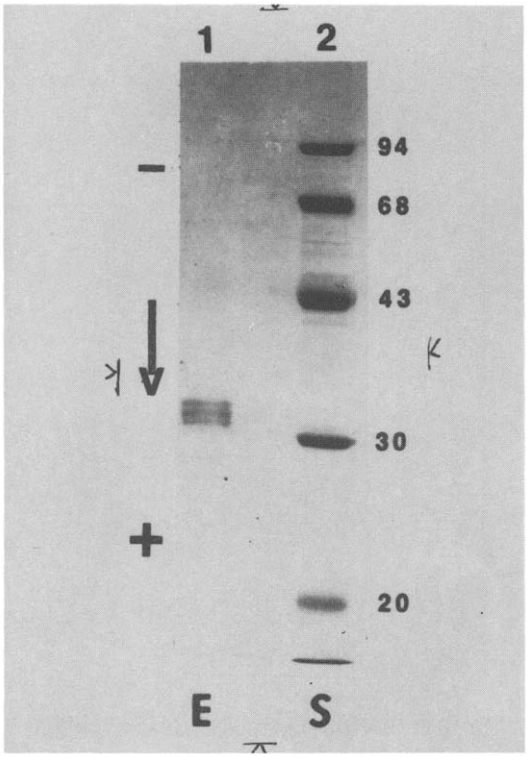

Fig. 4. SDS-PAGE of the HPLC-purified elastase in 8% polyacrylamide gel. Samples were reduced in 8 *M* urea and 0.1 *M* dithiotreitol. Lane 1: 50 μg elastase purified as described in Fig. 3; lane 2: protein standards of 94, 68, 43, 30, 20 and 14 kDA. The 14-kDA standard migrated with the dye front at the gel bottom.

DISCUSSION

There are several published procedures for purification of neutrophil elastase. The method of Baugh and Travis [1] employs use of a Sepharose-Trasylol aprotinin affinity column followed by CM-cellulose chromatography, while the method of Barrett [2] is a further modification of this method. This purification procedure reported here employs the speed and higher resolution of HPLC and of HPLC-specific resins. The procedure is novel in that the innate tendency of HPLC gel permeation columns to electrostatically bind proteins, a situation usually considered to be a problem due to carboxylate groups on the resin [10], is used to advantage here. By application of extract to the column at low ionic strength (20 m*M* NaCl, 10 m*M* sodium acetate) the attraction of cationic elastase with the carboxylic groups on the column is enhanced. Since the carboxyl groups have p*K* values in the pH 4–5 range, careful manipulation of the pH of the buffer should allow great resolution with this method. This method allowed the elastase in the extract to bind to the column and to be eluted in a relatively pure form (99% pure) by subsequent application of a salt gradient of 0.02–1 *M* NaCl. Such a method should work as well for many other proteins which may not be as cationic as elastase.

ACKNOWLEDGEMENT

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