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A RAPID METHOD OF PURIFICATION OF HUMAN GRANULOCYTE CATIONIC NEUTRAL PROTEASES: PURIFICATION AND FURTHER CHARACTERIZATION OF HUMAN GRANULOCYTE ELASTASE*

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Summary

Human granulocyte elastase (EC 3.4.21.-) was isolated and purified (yield = 62%, purity = 91—100%) by a new short procedure using affinity chromatography using phenylbutylamine covalently linked to Affi-Gel. The granulocyte elastase was found to have a molecular weight of 34 400 by sodium dodecyl sulphate gel electrophoresis and the molecular weight obtained from the amino acid composition was 34 970. The composition of elastase purified from normal leucocytes showed some significant differences from that of enzyme purified by others from leukemic leucocytes. The granulocyte elastase hydrolyzed typical pancreatic elastase substrates like Boc-Ala-ONp and Ac-$(Ala)_3$-Nan. The enzyme was also found to have a weak enzymatic activity in hydrolysing acetyl-L-phenylalanine-α-naphthyl ester, a typical chymotrypsin substrate. A monospecific antiserum raised against the purified enzyme gave a single precipitin line with the pure enzyme and also with crude granular extract, both lines being identical.

Introduction

Several cationic, neutral proteases including elastase-like and chymotrypsin-like enzymes have been identified in human neutrophilic granulocytes [1—

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** Permanent address: Department of Biochemistry, Tel Aviv University, Ramat-Aviv, Tel Aviv, Israel. Abbreviations: *N*-*t*-Butyloxycarbonyl-L-alanine-*p*-nitrophenyl ester, Boc-Ala-ONp; *N*-benzoyl-L-tyrosine ethyl ester, Bz-Tyr-OEt; *N*-acetyl-DL-alanine-α-naphthyl ester, Ac-DL-Ala-1-ONap; *N*-acetyl-L-phenylalanine-α-naphthyl ester, Ac-Phe-1-ONap; dimethyl sulfoxide, Me_2SO; 4-phenylbutylamine covalently bound to Affi-Gel, PhBuN-Affi-Gel.

7]. Interest in these enzymes has been stimulated by mounting evidence suggesting their participation in a variety of physiologic and pathologic events [8—10]. In the preceding paper [11], a method was described for the purification of human granulocyte chymotrypsin-like enzyme and some of the properties of this enzyme were identified. The present paper will describe the application of this same new method to the purification of human granulocyte elastase-like enzymes and will further characterize these enzymes. Although the synthetic substrates employed to monitor elastase purification are not absolutely specific for elastases, there is adequate reason to believe from earlier work of one of the present authors [4] that, when applied to extracts from leucocyte granules, they do indicate elastase activity.

Materials and Methods

Materials

N-Benzoyl-L-tyrosine ethyl ester (Bz-Tyr-OEt) and horseradish peroxidase were obtained from Sigma Chemical Company, St. Louis, Missouri. *N-t*-Butyloxycarbonyl-L-alanine-*p*-nitrophenyl ester (Boc-Ala-ONp) was from Cyclo Chemical Corp., Los Angeles, California and *N*-acetyl-DL-alanine-α-naphthyl ester (Ac-DL-Ala-1-ONap) from Fox Chemical Co., Los Angeles, California. 4-Phenylbutylamine was from Aldrich Chemical Co., Milwaukee, Wisconsin and Affi-Gel 10 from Bio-Rad Laboratories, Richmond, California. *N*-Acetyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide (Ac-$(Ala)_3$-Nan) was from Miles-Yeda, Rehovoth, Israel. Porcine elastase was from Worthington Biochemical Corp., Freehold, New Jersey. *N*-Acetyl-L-phenylalanine-α-naphthyl ester (Ac-Phe-1-ONap) was a gift from Dr L. Ornstein, Mt. Sinai School of Medicine, New York, New York. Fresh whole blood was obtained from Inter-County Blood Services, Rockville Centre, New York, a subsidiary of the New York Blood Center.

Methods

Purification of granulocyte elastase. The isolation of leucocytes, their disruption to yield granules and the extraction of granular proteins in 0.10 M NaCl were done as described in the preceding paper [11]. PhBuN-Affi-Gel was prepared according to Feinstein and Janoff [11] and affinity chromatography was carried out on a PhBuN-Affi-Gel column (10 cm × 1.2 cm) equilibrated with pH 7.5 0.01 M sodium phosphate buffer containing 0.5 M NaCl. The crude mixture of granular proteins was put on the column and the column was further washed with the same buffer (flow rate = 20 ml/h). After the elution of the bulk of the proteins with little or no elastase activity followed by the elution of a small amount of granulocyte chymotrypsin-like enzyme [11], the column was washed with phosphate buffer containing 1.0 M NaCl with or without Me_2SO to elute the granulocyte elastase. The pooled fractions were concentrated by UM-2 ultrafiltration, dialyzed against pH 7.5, 0.01 M sodium phosphate containing 0.10 M NaCl and lyophilized.

Enzymatic assays. All enzymatic assays were carried out at room temperature (20—22° C) and at pH 7.5 in 0.1 M sodium phosphate. A Gilford recording

spectrophotometer (Model 2400-S) was used. The hydrolysis of Boc-Ala-ONp (1% acetonitrile, $S_o = 5 \cdot 10^{-4}$ M) was determined at 400 nm according to Visser and Blout [12]. The hydrolysis of Ac-$(Ala)_3$-Nan (10% Me_2 So, $S_o = 5.1 \cdot 10^{-4}$ M) was determined at 410 nm according to Feinstein et al. [13]. The hydrolysis of Bz-Tyr-OEt (5% Me_2 SO, $S_o = 5 \cdot 10^{-4}$ M) was determined at 256 nm according to Hummel [14].

Gel electrophoresis studies. Cationic acrylamide disc gel electrophoresis was carried out according to Reisfeld et al. [15] with slight modification [16]. In the sodium dodecyl sulphate gel electrophoresis [11,17] horseradish peroxidase (M_r = 40 000) and soybean trypsin inhibitor (M_r = 21 500) were used as markers to calculate the molecular weight of the granulocyte elastase.

Amino acid analysis. The amino acid composition of the elastase was determined on a Beckman 120C Amino Acid Analyzer equipped with a computer integrator. Hydrolysis was carried out with 5.7 M HCl at 110° C for 24 and 48 h, after reduction with dithiothreitol. The values of the amino acids were mostly averaged and some were corrected for destruction during the acidic hydrolysis.

Protein determination. The Lowry method [18] was used for protein determination using bovine serum albumin for calibration. The protein concentration of porcine elastase was calculated from the absorbance at 280 nm ($A^{1\%}_{280} = 20.2$).

Preparation of antisera. 0.5 mg of purified elastase was dissolved in pH 7.5 phosphate buffer and emulsified with an equal volume of complete Freund's Adjuvant. Subcutaneous injections were made in an adult, male, New Zealand rabbit in the inguinal, axillary and cervical regions. One month later, the rabbit was boosted with 0.2 mg of purified enzyme, intravenously. Over the course of many months, several additional 0.1 mg intravenous booster injections of pure enzyme were administered about 7—10 days before bleedings. Serum was collected on several occasions and tested by double immunodiffusion or immunoelectrophoresis as will be described under Results. All sera were separately stored at —80° C, either as whole serum or as concentrated euglobulin fractions prepared by $(NH_4)_2 SO_4$ precipitation.

For preparation of antiserum to crude granular extract, 2.0 mg protein of the 0.1 M NaCl granule extract in 0.5 ml of phosphate buffered (pH 7.5) saline were diluted to 1.5 ml with buffered saline and emulsified in an equal volume of complete Freund's Adjuvant. Cervical and axillary subcutaneous injections were made into an adult, male albino rabbit. After one month, 0.8 mg protein of the original crude granular extract were administered intravenously as a booster and a similar dose was given again two weeks after the first booster. Eight days after the last dose, the rabbit was bled and the whole serum fraction stored at —80° C.

Results and Discussion

Differential salt extraction of human leucocyte granules. The procedure for achieving a crude separation of granulocyte chymotrypsin-like enzyme and elastase prior to affinity chromatography, was described in the preceding paper [11]. This step was achieved by differential NaCl extraction of the granules

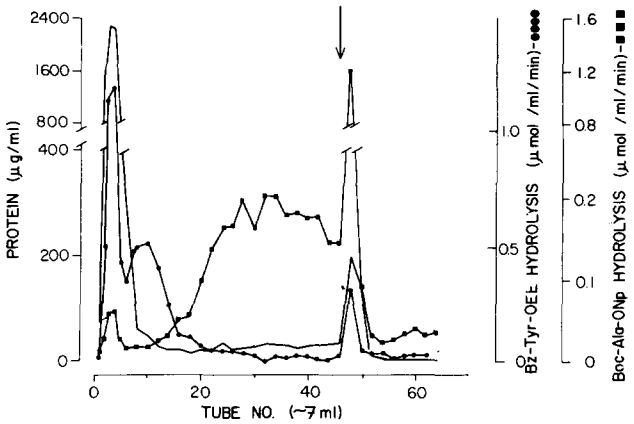

Fig. 1. Affinity chromatography of low salt (0.10 M NaCl) granular extract on PhBuN-Affi-Gel column. The PhBuN-Affi-Gel column (10 cm × 1.2 cm) was equilibrated and run in the cold with pH 7.5, 0.01 M $NaPO_4$ buffer containing 0.5 M NaCl. The sample was applied to the column in the same buffer. Buffer change (arrow) was to phosphate buffer containing 1.0 M NaCl and 20% Me_2SO. Flow rate, 20 ml/h. Substrate: Bz-Tyr-OEt, ●———●; Boc-Ala-ONp, ■———■.

giving rise to low salt (0.1 M) and high salt (1.0 M) solutions of granular proteins. The 0.1 M NaCl extracts were combined and concentrated by ultrafiltration on UM-2 Diaflo membranes, adjusted to give a final NaCl concentration of 0.5 M and were then loaded onto the PhBuN-Affi-Gel column.

Affinity chromatography of 0.1 M NaCl granule extract on PhBuN-Affi-Gel. Figs 1 and 2 represent two separate elastase purification runs with low salt extracts of leucocyte granules. In both cases, the bulk of granular proteins was eliminated in the run-through fractions, although some chymotrypsin-like activity (about 25% of the total recovered from the low salt extract) was lost with

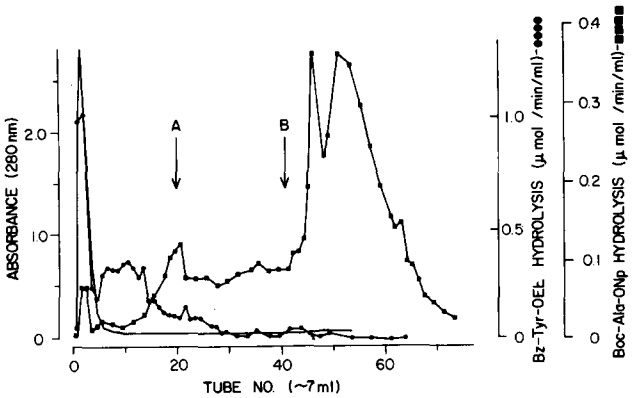

Fig. 2. Affinity chromatography of low salt (0.10 M NaCl) granular extract on PhBuN-Affi-Gel column. The PhBuN-Affi-Gel column (10 cm × 1.2 cm) was equilibrated and run in the cold with pH 7.5, 0.01 M $NaPO_4$ buffer containing 0.5 M NaCl. The sample was applied to the column in the same buffer. First buffer change (arrow A): same $NaPO_4$ buffer containing 1.0 M NaCl. Second buffer change (arrow B): 0—20% Me_2SO gradient in pH 7.5, 0.01 M $NaPO_4$ containing 1.0 M NaCl. Flow rate, 20 ml/h. Substrate hydrolysis: Boc-Ala-ONp, ■———■; Bz-Tyr-OEt, ●———●.

the run-through proteins. The remainder of chymotrypsin-like activity in the low salt extract was recovered by continued washing of the column with 0.5 M NaCl + 0.01 M phosphate buffer, pH 7.5. This material was concentrated by UM-2 Diaflo filtration and pooled with the 1.0 M granular salt extract for final purification of leucocyte chymotrypsin-like enzyme, as described in the preceding paper [11]. Thereafter, either of two procedures was followed in order to obtain purified elastase. In one procedure (Fig. 1), column washing with 0.5 M NaCl (pH 7.5) was continued until elastase activity (as monitored by Boc-Ala-ONp hydrolysis) began to appear in the column effluent. Washing with this buffer was then continued until elastase activity of effluent fractions began, in turn, to decrease. At that time, the column wash was changed to 1.0 M NaCl + 0.01 M phosphate buffer, pH 7.5 + 20% Me_2SO (Fig. 1, arrow). This wash eluted a sharp peak of additional elastase activity from the column, as shown in Fig. 1. A small amount of Bz-Tyr-OEt esterase activity also appeared in the peak elastase fraction eluted with Me_2SO as shown in the Fig., which was due to a small amount of leucocyte chymotrypsin that had remained bound to the column despite extensive earlier washing with 0.5 M NaCl. A more satisfactory procedure for recovery of purified elastase is shown in Fig. 2. In this method, column washing with 0.5 M NaCl (pH 7.5) was stopped as soon as the bulk of chymotrypsin-like activity had passed through the column, and a 1.0 M NaCl (pH 7.5) wash without Me_2SO was begun (see arrow A in Fig. 2). This wash was continued until Bz-Tyr-OEt esterase activity of the column effluent was no longer detectable, but before a significant amount of elastase activity had been eluted from the column. Thereafter, a Me_2SO gradient in 1.0 M NaCl (pH 7.5) was begun (arrow B in Fig. 2). Under these conditions, the bulk of elastase eluted with that portion of the gradient between 5 and 12% Me_2SO and was obtained completely free of chymotrypsin-like activity. All traces of the latter had been eluted in the preceding 1.0 M NaCl wash. Little protein could be detected in the diluted elastase fractions recovered by the Me_2SO gradient method, where A_{280} was the monitor employed. Protein was detected in the elastase fractions recovered by the first method, where the more sensitive Lowry assay was employed as monitor.

Some variation in the patterns of protein bands obtained by acrylamide gel electrophoresis was observed, when the elastase eluted with 0.5 M NaCl (Fig. 1) was compared to that eluted with Me_2SO plus NaCl (Figs 1 and 2). The minor, slower-moving elastase isoenzymes were selectively enriched in the 0.5 M NaCl effluent, whereas the major, rapidly-moving isoenzyme was more pronounced in the gel pattern given by Me_2SO-eluted elastase (see gels 1 and 2A of Fig. 4). These isoenzymes of elastase have previously been described in earlier work from this laboratory [4] as well as by other workers [5,19].

Tables I and II summarize the purification of leucocyte elastase according to the two procedures outlined above. "Early elastase" in the tables refers to enzyme recovered prior to introduction of Me_2SO into the column wash. "Elastase" in the tables refers to enzyme eluted with Me_2SO. It should be noted that degree of purification at each step is based on the specific activity of the starting granular extract, whereas the latter value already represents a several 100-fold purification of enzyme from whole blood. Only Me_2SO-eluted "elastase" was used for determination of molecular weight and for amino acid

TABLE I

SUMMARY OF PURIFICATION OF GRANULAR ELASTASE USING THE FIRST METHOD (NO GRADIENT)

Step	Protein (mg)	Boc-Ala-ONp hydrolysis		Purification**
		Units*	% Yield	
Crude granular extract put on PhBuN-Affi-Gel column (Fig. 1)	51.3	86.4	100	1
Pooled fractions from column				
Early elastase (tubes No. 15—29)	n.d.	16.7	19.3	—
Elastase (tubes No. 30—54)	n.d.	37.9	43.9	—
Lyophilized material				
Early elastase	1.86	19.8	22.9	7.4
Elastase	4.27	33.8	39.1	5.6

* Unit, one μmol Boc-Ala-ONp hydrolyzed per min.
** See text for clarification.

and immunochemical analyses. The specific preparation used for amino acid analysis had no detectable "slow elastase-like esterase" (for explanation, see discussion of zymograms in the following section).

Electrophoretic monitoring of purification. Fig. 3 shows the overall pattern of protein bands (Buffalo Black stain) given by the 0.10 M NaCl granular extract (gel 1A) and by the mixture of proteins appearing in the run-through fractions of the PhBuN-Affi-Gel column (gel 2). Also shown are the zymograms obtained when gels containing 0.1 M granule extract were stained with Ac-DL-Ala-1-ONap for elastase activity (gel 1B) or with Ac-Phe-1-ONap for chymotrypsin-like activity (gel 1C). Fig. 4 (gel 1) shows the Buffalo Black staining pattern given by the purified elastase isoenzymes eluted from PhBuN-Affi-Gel by 0.5 M NaCl ("early elastase", see preceding section) as well as the Buffalo Black pattern (gel 2A) given by purified elastase isoenzymes eluted with Me_2SO + NaCl ("elastase", see preceding section). The existence of multiple

TABLE II

SUMMARY OF PURIFICATION OF GRANULAR ELASTASE USING THE SECOND METHOD (Me_2SO GRADIENT)

Step	Protein (mg)	Boc-Ala-ONp-hydrolysis		Purification**
		Units*	% Yield	
Crude granular extract put on PhBuN-Affi-Gel column (Fig. 2)	59.2	84.2	100	1
Pooled fractions from column				
Early elastase (tubes No. 15—29)	n.d.	7.1	8.4	—
Elastase (tubes No. 30—74)	n.d.	45	53.4	—
Lyophilized material				
Early elastase	n.d.	4.0	4.7	—
Elastase	2.66	24.3	28.9	6.4

* Unit, one μmol Boc-Ala-ONp hydrolyzed per min.
** See text for clarification.

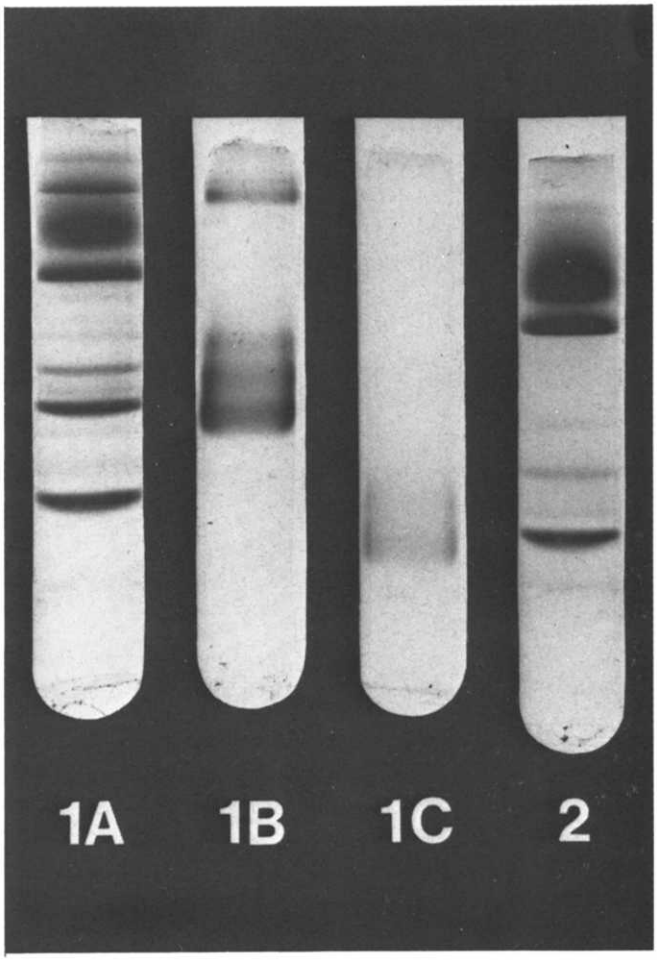

Fig. 3. Acrylamide disc gel electrophoretograms. Low salt (0.10 M NaCl) crude granular extract (1) and "runthrough" fraction from PhBuN-Affi-Gel chromatography (2). Migration from top to bottom. 1A and 2, 174 μg protein each, Buffalo Black Stain; 1B, 116 μg protein, Ac-DL-Ala-1-ONap stain; 1C, 116 μg protein, Ac-Phe-1-ONap stain. See text for details.

elastase isoenzymes in the human leucocyte granule has now been confirmed in several independent studies [4,5,19]. Also shown in Fig. 4 are gel zymograms obtained when purified elastase isoenzymes (Me_2 SO-eluted) were stained with Ac-DL-Ala-1-ONap (2B) or Ac-Phe-1-ONap (2C). The absence of contaminating chymotrypsin is obvious from an inspection of the latter gel (2C), which contained considerably more protein than did gel 2B (see Fig. legend for details). Gel 2C also shows that the leucocyte elastases themselves have weak activity against the chymotrypsin substrate.

In addition to the elastase isoenzymes with intermediate electrophoretic mobility, a slow-migrating component with enzymatic activity against Ac-DL-Ala-1-ONap is also evident in some of the zymograms shown in Figs 3 and 4. This "slow elastase-like esterase" has not been isolated or characterized to date, but it appears to be present in greater amounts, relative to the other elastases,

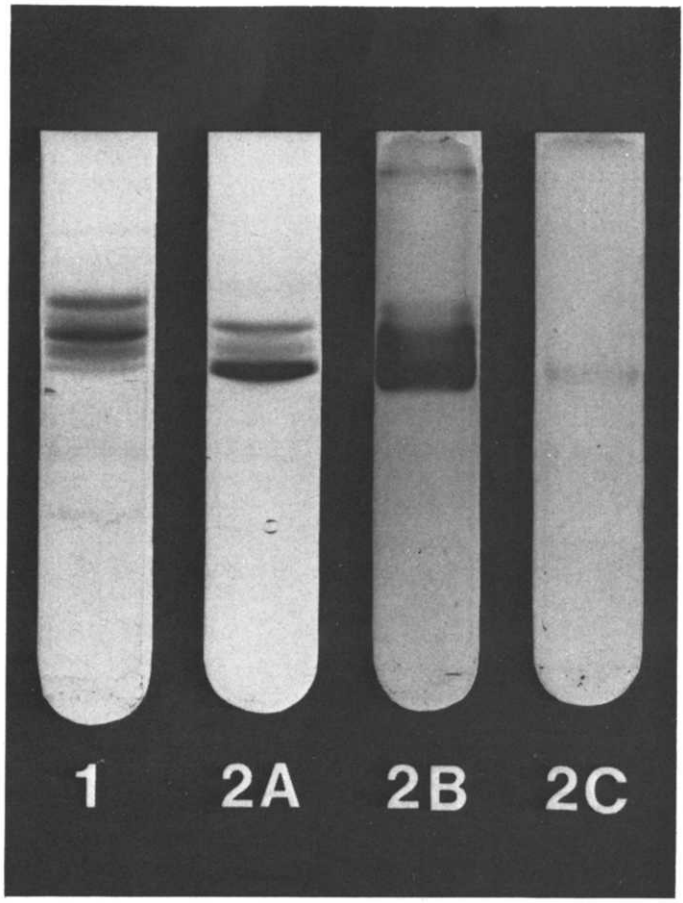

Fig. 4. Acrylamide disc gel electrophoretograms. Purified elastase fractions. Early elastase (1); Elastase (2). Migration from top to bottom. 42.5 μg of protein, Buffalo Black stain (1); 45 μg protein, Buffalo Black stain (2A); 12.9 μg protein, Ac-DL-Ala-1-ONap stain (2B); 43 μg protein, Ac-Phe-1-ONap stain (2C). See text for details.

in granular extracts prepared from leukemic or pre-leukemic granulocytes or normal bone marrow cells (unpublished observations of the present authors). It may therefore represent a precursor or zymogen-form of the more rapidly migrating isoenzymes. Studies are now in progress in our laboratory to investigate this possibility.

Fig. 5 shows densitometric scans of gels 1A in Fig. 3 and 2A in Fig. 4. Protein quantities included in the gels are given in the accompanying legends to Figs 3 and 4. When standardized to equal protein loads and corrected for differences in the optical density scales used during scanning, the figure reveals about an eight-fold selective enrichment of elastase after purification. This purified elastase constituted 100% of the protein present in the gel pattern.

Molecular weight estimation of purified elastase. Samples of purified enzyme were denatured by heating at 56°C for 90 min in the presence of 1% sodium dodecyl sulphate, 0.04 M dithiothreitol and 0.001 M EDTA and were then subjected to electrophoresis at pH 8.6 in 12% acrylamide gels containing

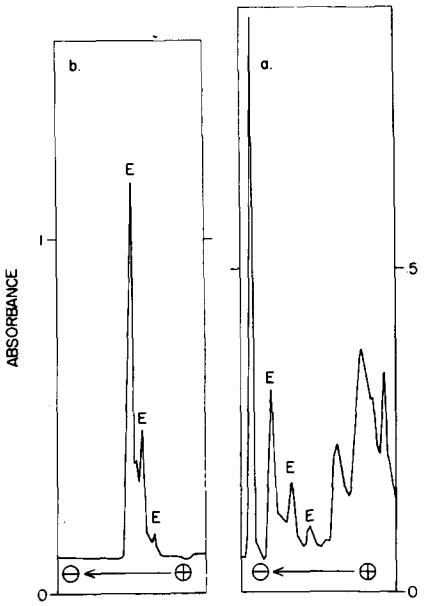

Fig. 5. Scans of acrylamide disc gel electrophoretograms. a, low salt (0.10 M NaCl) crude granular extract, 130 μg protein; b, elastase, 45 μg protein. Both stained with Buffalo Black stain. See text for details.

TABLE III

AMINO ACID ANALYSIS AND COMPOSITION OF HUMAN GRANULOCYTE ELASTASE

The amino acid composition we report herein was calculated assuming 3 mol of lysine residues per mol protein. The results are also expressed as mol % for comparison with previously published data by Ohlsson and Olsson [5].

Amino acid	nmol in analysis	mol/mol	% mol amino acids	
			a*	b**
Lysine	10.3	3	1.0	0.5
Histidine	31.0	9	3.0	2.8
Arginine	92.5	27	9.0	11.7
Aspartic acid	99.6	29	9.7	9.6
Threonine	29.2	9	3.0	2.8
Serine	58.2	17	5.7	5.2
Glutamic acid	75.8	22	7.3	7.0
Proline	35.2	10	3.3	7.4
Glycine	99.0	29	9.7	11.2
Alanine	90.2	27	9.0	11.1
Half-cysteine	31.5	9	3.0	+
Valine	74.4	22	7.3	11.4
Methionine	29.7	9	3.0	0.7
Isoleucine	61.5	18	6.0	4.2
Leucine	95.8	28	9.3	9.7
Tyrosine	43.8	13	4.3	1.1
Phenylalanine	66.2	19	6.3	3.9
Tryptophan	n.d.	n.d.	n.d.	n.d.
Total		300	100	100

* = this study.

** = data from Ohlsson and Olsson [5], numbers refer to "Elastase I".

0.25% sodium dodecyl sulphate. The gels were calibrated with crystalline horseradish peroxidase (M_r = 40 000) and crystalline soybean trypsin inhibitor (M_r = 21 500), which had first been similarly denatured. An estimated M_r of 34 400 was calculated for the leucocyte elastase by this method. This value agrees well with the M_r of 33 000–36 000 estimated by Ohlsson and Olsson [5] for leucocyte elastase, but is considerably larger than the values given by Taylor and Crawford [20] or Baugh and Travis [21] of 22 000 and 26 000, respectively.

Amino acid analysis of purified elastase. Table III presents the results of our amino acid analysis of purified leucocyte elastase. On the basis of the compositional analysis, a M_r of 34 970 was calculated which corresponds closely to the value obtained from sodium dodecyl sulphate gel electrophoresis given above. The amino acid analysis presented in Table III, which is based on enzyme obtained from normal leucocytes, can now be compared with data previously published by Ohlsson and Olsson [5] for human leucocyte elastase derived from leukemic cells. Such a comparison shows a number of significant differences in the amino acid composition of the enzymes prepared from these two different sources. In our preparation, lysine, methionine, isoleucine, tyrosine and phenylalanine were present in significantly larger proportion than in the leukemic enzyme preparation, whereas the opposite was true for proline and valine. It should be noted, however, that elastase purified from leukemic

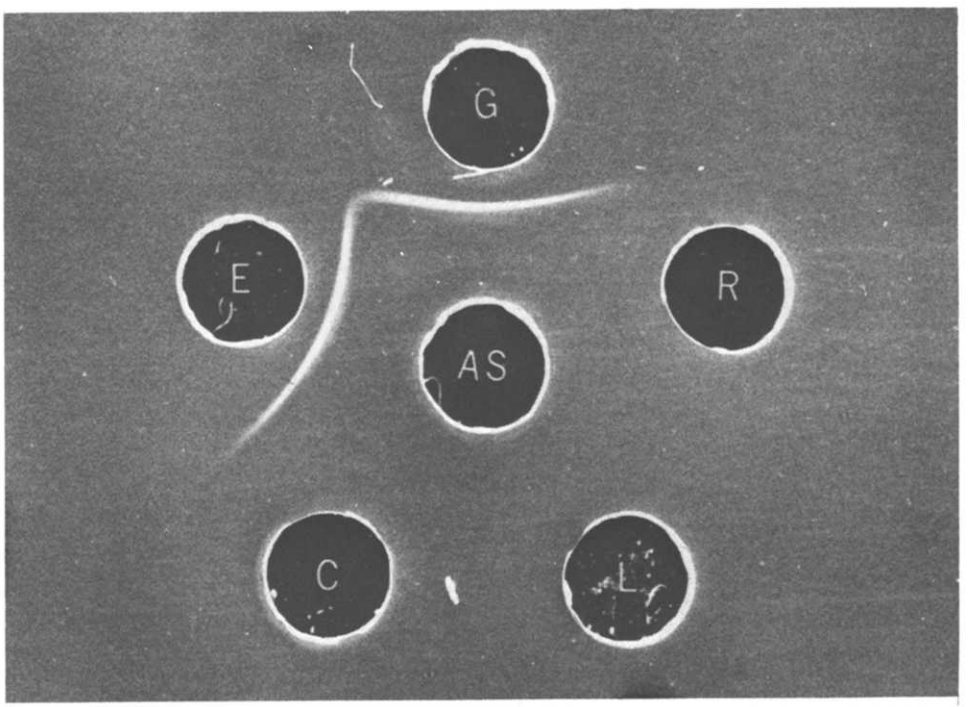

Fig. 6. Double immunodiffusion between rabbit antiserum to leucocyte elastase and different components of the granular extract. AS = anti-elastase antiserum, euglobulin-enriched fraction, 10 µl; E = purified leucocyte elastase, 2.8 µg; G = crude granular extract (0.1 M NaCl), 18.7 µg; R = run-through proteins of crude granular extract (PhBuN-Affi-Gel fractions), 17.4 µg; L = purified leucocyte lysozyme, 2.0 µg; C = purified leucocyte chymotrypsin-like enzyme (ref. 11), 3.9 µg.

cells was observed to be immunologically identical to that obtained from normal leucocytes [5]. Further study will be required to explain these differences in the amino acid compositions of elastases prepared from normal and leukemic cells.

Hydrolysis of synthetic substrates by purified leucocyte elastase. The purified human leucocyte enzyme hydrolyzed several typical porcine pancreatic elastase substrates such as Boc-Ala-ONp and Ac-$(Ala)_3$-Nan. However, while the pancreatic enzyme hydrolyzed Boc-Ala-ONp at twice the relative rate that it cleaved Ac-$(Ala)_3$-Nan, this ratio was 20-fold higher in the case of the leucocyte elastase. Thus, Ac-$(Ala)_3$-Nan is a relatively poor substrate for the leucocyte elastase. As mentioned earlier, the leucocyte elastases also possessed weak activity against Ac-Phe-1-ONap, a synthetic substrate for chymotrypsin (see gel 2C in Fig. 4).

Immunochemical analysis of purified elastase. Figs 6 and 7 show the results of double immunodiffusion tests employing purified leucocyte elastase and other granule components vs. rabbit antisera raised against the purified enzyme (Fig. 6) or against crude granular extract (Fig. 7). The unispecific character of the antiserum raised to purified leucocyte elastase is evident from Fig. 6. It can be seen that this antiserum gave single lines of identity against pure elastase and crude granular extract (0.1 M NaCl), but failed to react against purified leucocyte chymotrypsin-like enzyme, purified leucocyte lyso-

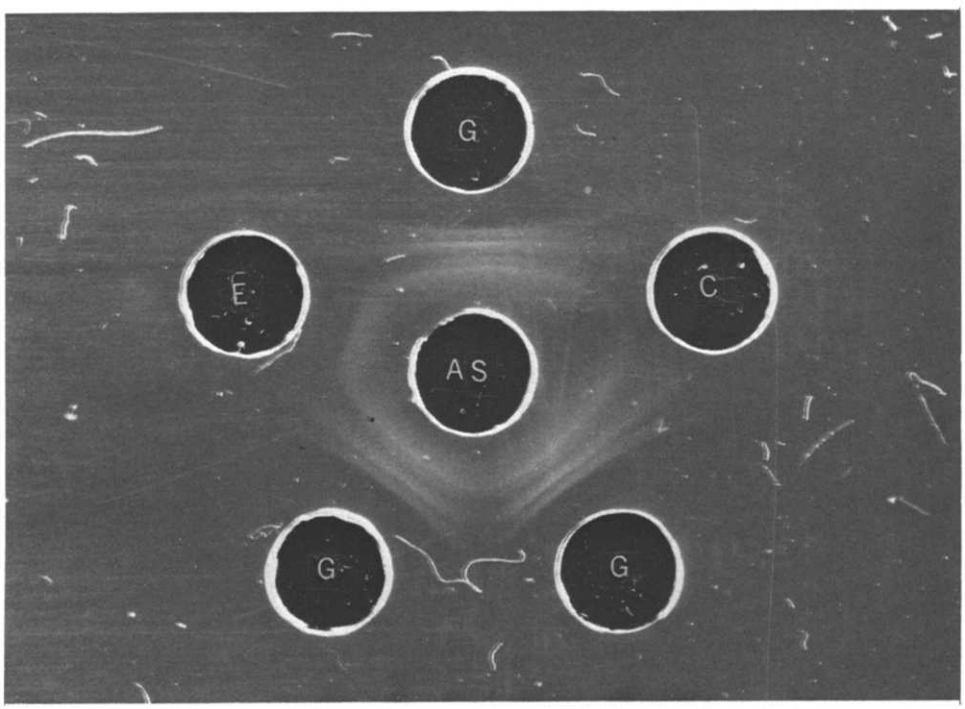

Fig. 7. Double immunodiffusion between rabbit antiserum to crude granular extract and crude granular extract or purified enzymes. AS = anti-crude granular extract (0.1 M NaCl) antiserum, whole serum, 10 μl; G = crude granular extract (0.1 M NaCl), 18.7 μg; E = purified leucocyte elastase, 2.8 μg; C = purified leucocyte chymotrypsin-like enzyme (ref. 11), 3.9 μg.

zyme or the PhBuN-Affi-Gel fraction containing the run-through proteins of the crude granule extract. (See Fig. legend for protein concentrations used.) In a separate experiment, not shown here, crude granular extract was first subjected to electrophoresis in an acrylamide disc gel and the gel was sliced uniformly throughout its length. Individual gel slices were then set into wells cut into an agarose plate and double diffusion allowed to proceed against the anti-elastase antiserum. Only those gel slices containing the elastase isoenzymes (as determined from a parallel acrylamide gel stained with Buffalo Black) gave a precipitin line.

Fig. 7 shows that antiserum raised to crude leucocyte granular extract (0.1 M NaCl) gave four precipitin lines when run against the crude extract. One of these lines was identical to the precipitin line which formed between the same antiserum and purified elastase placed in an adjacent well. The other three lines developed closer to the well containing the granular antigens suggesting that components of the granular extract with M_r larger than that of elastase were involved. It can also be noted in Fig. 7 that no precipitin reaction occurred between the anti-granular extract antiserum and purified leucocyte chymotrypsin-like enzyme [11]. This was probably a result of the relatively small amount of chymotrypsin-like enzyme present in the 0.1 M NaCl granular extract used to immunize the rabbit.

In conclusion, the foregoing results show that elastase-like enzymes can also be purified from human, neutrophil leucocyte granules by differential NaCl extraction followed by affinity chromatography on PhBuN-Affi-Gel. The method is similar to that described in the preceding paper [11] for purification of the leucocyte chymotrypsin-like enzyme. Human granulocyte elastase has been demonstrated to attack a variety of connective tissue structures including arterial elastin [1], vascular endothelium [22] and cartilage matrix [23,24], and also to participate in the digestion of *E. coli* proteins by neutrophil granules in vitro [25]. Isolation and characterization of leucocyte elastases, as well as the chymotrypsin-like enzyme, should permit further extension of our knowledge of human leucocyte functions, under both normal and pathological conditions.

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