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PURIFICATION AND CHARACTERISATION OF AN 'ELASTASE-LIKE' ENZYME FROM RABBIT POLYMORPHONUCLEAR LEUCOCYTES

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Summary

An enzyme with proteolytic activity has been isolated from the subcellular granules of rabbit polymorphonuclear leucocytes. Purification of the enzyme involved extraction of the granule membranes with 0.01 M sodium phosphate buffer, pH 7.4, containing 1 M NaCl and 0.1% Triton X-100, followed by gel exclusion chromatography on Sephadex G-75. The enzyme hydrolysed *p*-nitrophenol-*N*-*tert*-butyloxylcarbonyl-L-alaninate, a synthetic substrate for elastase, but failed to hydrolyse elastin. The enzyme also hydrolysed azo-albumin with a pH optimum between 7.5 and 8.5. Inhibition studies indicated that the enzyme was a serine proteinase (EC 3.4.21.—) and it was found to have an apparent molecular weight of 25 000 by polyacrylamide gel electrophoresis. The purified enzyme behaved as a single protein on SDS-polyacrylamide gel electrophoresis, had a single isoelectric point at pH 5.9, yet showed multiple components on centrifugation.

Introduction

Polymorphonuclear leucocytes are thought to participate in the pathogenesis of connective tissue diseases, since they invade tissues in response to local immune-complex formation [1]. Such behaviour has led to the suggestion that leucocytes might release proteinases which degrade connective tissue components, prompting an examination of the proteinases present in these cells. Human polymorphs have been shown to contain an elastase, a collagenase, and a chymotrypsin-like enzyme, cathepsin G (EC 3.4.21.20) [2—4]. There have

been few studies of the proteinase present in rabbit polymorphs, although this species has been used extensively in studies of disorders such as immune-complex and nephrotoxic serum nephritis. The cells from rabbits are known to contain acid proteinases [5], but their neutral proteinase activity is 10—30-times lower than that of human polymorphs [6]. Recent studies have shown that rabbit polymorphs contain a neutral proteinase which acts on histone [7], a collagenase [8] and an 'elastase' [6], so described since the enzyme hydrolysed the substrate *p*-nitrophenol-*N-tert*-butyloxylcarbonyl-L-alaninate (Np-Boc-Ala), a synthetic substrate for elastase. Here we report the isolation and characterisation of this 'elastase-like' enzyme from rabbit polymorphs.

Materials and Methods

Rabbits, New Zealand White (either sex) weighing 2—2.5 kg, were obtained from the Oxford University farm. Chemicals were obtained commercially: *p*-nitrophenol-*N-tert*-butyloxylcarbonyl-L-alaninate (Np-Boc-Ala), *N*-benzoyl-L-tyrosine ethyl ester, elastin-orcein, azo-albumin, horse immunoglobulin G, cytochrome *c* from horse heart, trypsin inhibitor from soya beans, phenylmethylsulphonyl fluoride and Triton X-100 were purchased from the Sigma (London) Chemical Co. Ltd. (U.K.). Oyster glycogen was obtained from B.D.H. Chemicals Ltd. (Poole, U.K.), ampholines from Bio-Rad Laboratories (U.K.) and Sephadex G-75 from Pharmacia, London (U.K.).

Preparation of fractions from leucocyte granules. Rabbit polymorphonuclear leucocytes were harvested from peritoneal exudates collected after intraperitoneal injection of 200 ml 0.1% (w/v) oyster glycogen in 0.15 M NaCl [9]. The leucocyte preparations, which contained 2—5% of other cells, were washed in 0.34 M sucrose and then disrupted by homogenization in the same solution until all the cells were ruptured as judged by phase contrast microscopy. Any intact cells and the nuclei were removed by centrifugation at $600 \times g$ for 10 min; the granule fraction was recovered from the supernatant by centrifugation at $12\,000 \times g$ for 20 min. The granule pellet, comprised of both large electron dense granules and granules which appeared to contain particles, was suspended in 10—15 ml 0.01 M sodium phosphate buffer (pH 7.4). The suspension was frozen in solid CO_2/acetone mixture and thawed at room temperature three times before centrifugation at $30\,000 \times g$ for 30 min. The pellet (the granule membranes) was extracted at 0°C with 0.01 M sodium phosphate buffer (pH 7.4)/1 M NaCl/0.1% Triton X-100 for a period of 20 min; the extraction procedure was repeated twice more and the supernatants were combined as the 'membrane extract'. This treatment released 70—85% of the Np-Boc-Ala esterase activity from the granule membranes.

Enzyme assays. Esterase activity was measured by following the hydrolysis of Np-Boc-Ala [10]. The assay mixture, 2.4 ml, contained 1.0 μmol Np-Boc-Ala in 0.05 M sodium phosphate buffer (pH 6.5) and 1 M NaCl; enzyme was added and the reaction followed by measuring the change in A_{347nm} using a blank minus enzyme. For convenience the (0.01 M) substrate was initially dissolved in acetonitrile and (0.1 ml) this solution was added to the buffer (2.4 ml). 1 M NaCl gave optimal activation of the esterase under these conditions.

Assays were carried out at 25°C under zero-order conditions; activity is expressed as μmoles *p*-nitrophenol released per min. *p*-Nitrophenol was the standard.

Elastase activity was measured by determining the amount of soluble dye released from orcein-impregnated elastin [11]. Elastin-orcein was suspended in 0.05 M Tris-HCl buffer (pH 8.6) at a concentration of 20 mg/ml, enzyme was added and incubation with constant shaking allowed to proceed for periods of up to 30 h at 37°C.

Cathepsin G activity was determined by the method of Kang and Fuchs [12] using *N*-benzoyl-L-tyrosine ethyl ester as substrate. Collagenase activity was measured using the method described by Harris and Cartwright [13] for bovine tendon collagen; in some experiments renal basement membrane [14] was used as an alternate substrate.

Proteolytic activity was measured using 5 mg azo-albumin suspended in 2 ml 0.01 M sodium phosphate buffer (pH 7.4) containing 1 M NaCl, enzyme was added and the mixture incubated for 18 h at 37°C. The reaction was stopped by the addition of 1 ml 5% (w/v) trichloroacetic acid, the precipitated azo-albumin was removed by centrifugation and the absorbance (A_{440nm}) of the supernatant measured. Activity is expressed as μg of azo-albumin hydrolysed per h.

Studies with inhibitors. Antitrypsin inhibitor from soya beans was dissolved in 0.05 M sodium phosphate buffer (pH 6.5), 1 M NaCl. Known amounts of enzyme were mixed with the inhibitor and the mixture was allowed to stand at room temperature for 5 min; Np-Boc-Ala esterase activity was measured immediately afterwards. The effects of EDTA and $CaCl_2$ (5—20 mM) were examined following preincubation with the enzyme for 5 min at room temperature. Phenylmethylsulphonyl fluoride was dissolved in isopropanol to give a concentration of 100 mM and preincubated with the enzyme, final concentration 1 mM, for 16 h at 4°C before Np-Boc-Ala esterase activity was measured. Controls were incubated similarly in all cases.

Enzyme purification. The membrane extract was concentrated by ultrafiltration using YM 10 membranes (Amicon Ltd., High Wycombe, U.K.) to a protein concentration of 5—7 mg/ml. Samples of the concentrate were applied to a column of Sephadex G-75, 86 × 1 cm, equilibrated and eluted with 0.01 M sodium phosphate buffer, pH 7.4, containing 1 M NaCl; fractions were collected at a flow rate of 0.5 ml/min. Fractions were assayed for protein content (A_{280nm}) and for Np-Boc-Ala and azo-albumin hydrolysing activity. The column was calibrated with immunoglobulin G, bovine serum albumin, and cytochrome *c*. Fractions containing Np-Boc-Ala hydrolysing activity were pooled, concentrated by ultrafiltration as above, and rechromatographed under the same conditions.

Protein determination. The method of Lowry et al. [15] was used with bovine serum albumin as standard.

Polyacrylamide gel electrophoresis. Selected fractions from the Sephadex column were pooled and examined by polyacrylamide gel electrophoresis using gels which were prepared from 10% acrylamide, 0.01% bis-acrylamide in 0.1 M sodium phosphate buffer, pH 7.2, containing 1% SDS. Protein samples were heated for 5 min at 100°C in 0.01 M sodium phosphate buffer, pH 7.2, contain-

ing 1% SDS and 1% mercaptoethanol, before electrophoresis [16]. Immunoglobulin G, bovine serum albumin, myoglobin and cytochrome *c* were used as standards.

Isoelectric focusing gel electrophoresis. The procedure used was that described by O'Farrell [17]. Samples of the proteinase containing 5 μg and 25 μg protein were isofocused at 400 V for 18 h. Gels were extracted from the running tubes, immersed in 15% trichloroacetic acid for 30 min to fix proteins and then in a solution of 10% (v/v) acetic acid/ethanol for 24 h to elute ampholines. Gels were subsequently stained for proteins.

The pH gradient within gels was determined by cutting an unfixed gel into 0.5-cm sections, eluting the ampholines into 200 μl water and measuring the pH of the solution.

Amino acid analysis. Amino acids were estimated by the method of Spackman et al. [18]. Duplicate samples were hydrolysed in 6 N hydrochloric acid in vacuo at 105°C for 24 h before analysis.

Analytical ultracentrifugation. Sedimentation equilibrium [19] was used to determine the homogeneity of the purified protein. Centrifugation was carried out at 18 000 and 24 000 rev./min at 21°C using a Beckman model E analytical ultracentrifuge with optical scanning at 280 nm. Protein concentration was 0.8 mg/ml; equilibrium was reached within 24 h. For calculations of molecular weight, partial specific volume was taken as 0.73 $cm^3 \cdot g^{-1}$ calculated from the composition (Table IV).

Results

Freezing and thawing of the granule fraction in 0.01 M sodium phosphate buffer released approx. 40% of the total protein although most of the Np-Boc-Ala esterase activity remained bound to the granule membrane. Extraction of the membranes with 0.01 M sodium phosphate buffer, pH 7.4, containing 1 M NaCl and 0.1% (v/v) Triton X-100 released 70—85% of this bound activity. Enzyme and protein recoveries are shown in Table I.

It was found that increasing the concentration of NaCl in the assay increased the rate of Np-Boc-Ala hydrolysis; maximal activation, a 3-fold increase in specific activity, was achieved at 1 M concentration. The degree of activation was most marked with the 1 M NaCl extract of the granule membranes, lesser

TABLE I

DISTRIBUTION OF PROTEIN AND Np-Boc-Ala HYDROLYSING ACTIVITY IN THE GRANULE FRACTION AND IN SUB-FRACTIONS FROM RABBIT POLYMORPHONUCLEAR LEUCOYTES

These values are from one experiment. In different experiments yields of leucocytes varied, making it impossible to aggregate the results. All experiments showed similar increases in specific activities and similar percentage protein recoveries. Activity is expressed as μmoles of *p*-nitrophenol released per min.

Sample	Protein (mg)	%	Total activity	%	Specific activity
Granules	43	100	6762.0		157.2
Lysate	19	44	731.5	12.2	38.2
Membrane extract	16.5	38	4276.0	71.1	259.5
Extracted membranes	7.8	18	1002.0	16.6	128.4

activation being obtained with other fractions (Fig. 1); the extracted enzyme showed an increase in specific activity of 1.6-fold under the assay conditions. A similar pattern of activation by NaCl was also observed when azo-albumin was used as substrate.

Azo-albumin was used to determine the pH dependence of the proteolytic activity of the membrane extract giving an optimum between pH 7.5—8.5 (Fig. 2). Np-Boc-Ala hydrolysing activity increased with pH up to pH 8.0, but could not be measured above this value due to the spontaneous hydrolysis of the substrate.

Cathepsin G activity could not be detected in any fraction, at any NaCl concentration.

Sephadex column chromatography of the membrane extract yielded a significant purification of the Np-Boc-Ala esterase. The extract resolved into a number of peaks (Fig. 3) with the Np-Boc-Ala and azo-albumin hydrolysing activity occurring in fractions 24—29, the peak of activity eluting slightly ahead of cytochrome *c*. Table II shows the results of this step; it should be noted that the purification factor is based on the specific activity of the whole granules and not on the initial homogenate.

Purity

When the fractions corresponding to the Np-Boc-Ala esterase peak (Fig. 3) were pooled, concentrated and subjected to electrophoresis on SDS-polyacrylamide gels, one major and a few very faint minor bands were seen (Fig. 4). The presence or absence of mercaptoethanol made no difference to the band pat-

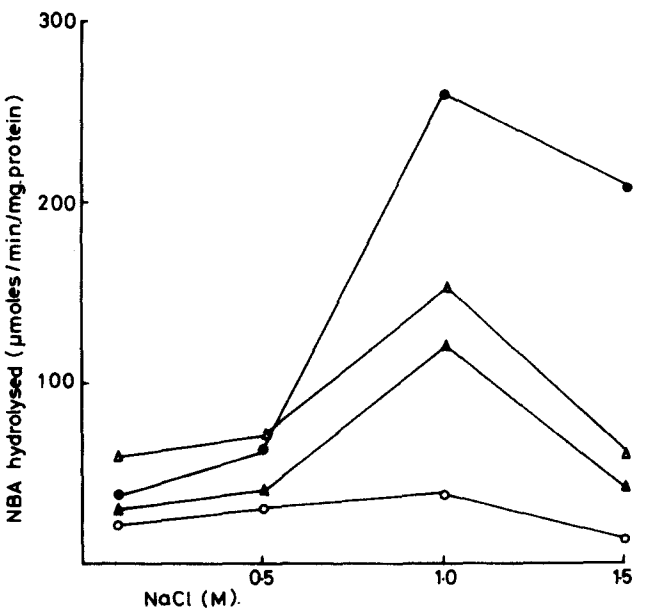

Fig. 1. The effect of NaCl concentration on the Np-Boc-Ala (NBA) hydrolysing activity of granule fractions: ●——●, membrane extract; △——△, whole granules; ○——○, granule lysate; ▲——▲, extracted membranes.

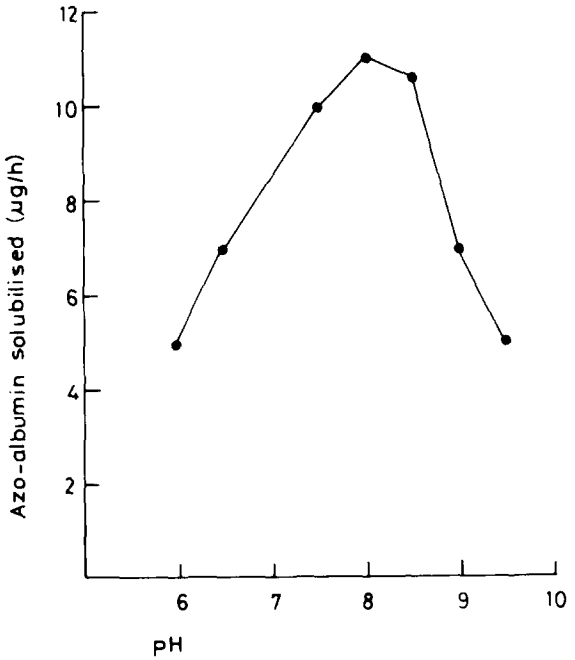

Fig. 2. Dependence of azo-albumin hydrolysing activity on pH: activity is expressed as μg of azo-albumin solubilised per h at 37°C.

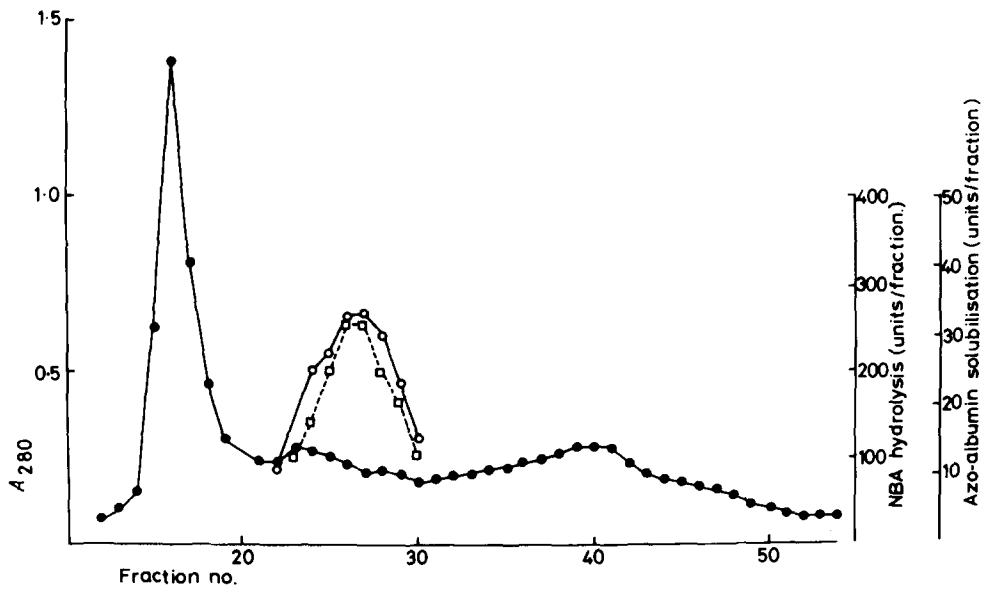

Fig. 3. Gel filtration of the membrane extract on Sephadex G-75. The column (86 × 1 cm) was equilibrated and eluted with 0.01 M sodium phosphate buffer, pH 7.4, containing 1 M NaCl. Fractions (2-ml) were sampled for protein (A_{280}) (●——●), Np-Boc-Ala (NBA) hydrolysing activity (□——□) and activity against azo-albumin (○——○).

TABLE II

SUMMARY OF PURIFICATION OF LEUCOCYTE Np-Boc-Ala ESTERASE

The values are from one experiment; in different experiments the yields of cells varied, making it impossible to average results. All experiments showed similar increases in specific activities and similar percentage protein recoveries. Np-Boc-Ala was used as the substrate.

Step	Total protein (mg)	Total units (μmol/min)	Purification
Whole granules	13.0	2049	1.0
Membrane extract	5.0	1613	2.0
Pooled fractions 24—28	0.98	1071	6.9
Pooled fractions from second column cycle	0.61	700	7.3

tern. The fractions displaying Np-Boc-Ala esterase activity were pooled, concentrated by ultrafiltration and rechromatographed on the Sephadex G-75 column. This procedure removed the minor contaminants (Fig. 4). Gel electrophoresis revealed that the enzyme had an apparent molecular weight of 25 000; the enzyme peak eluted from the Sephadex column at a position equivalent to a molecular weight of 25 000.

A sample of the purified enzyme in 0.01 M sodium phosphate buffer (pH 7.4)/1 M NaCl, was centrifuged in an analytical centrifuge at two speeds, 18 000 and 24 000 rev./min at 21°C. Analysis of the results demonstrated heterogeneity of the protein with species of molecular weight 26 000, 51 000, 82 000 and 98 000. These molecular species were present in material which

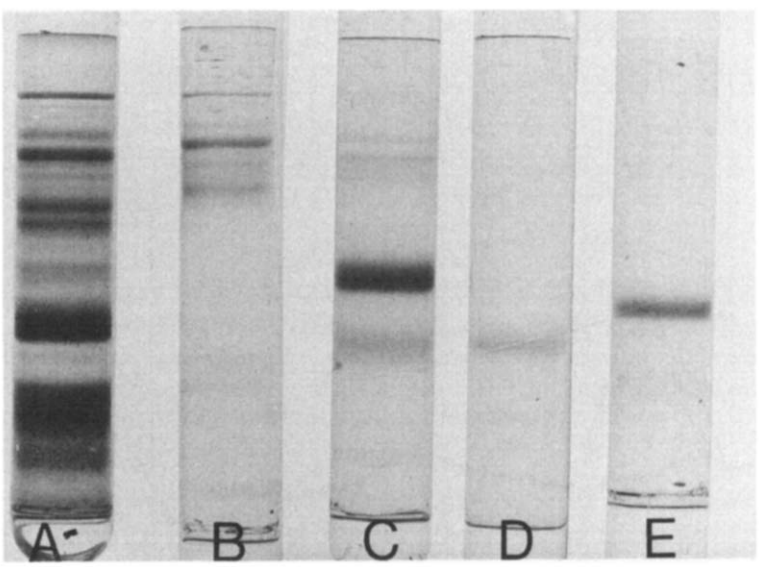

Fig. 4. Polyacrylamide gel electrophoresis of membrane extract and protein peaks from Sephadex column. Polyacrylamide electrophoresis gels were run in the presence of SDS and stained for protein with Coomassie brilliant blue R. (A) Membrane extract; (B) fractions 19—21 (Fig. 3); (C) fractions 25—28 (Fig. 3); (D) fractions 36—42 (Fig. 3) and (E) single protein peak from second cycle of Sephadex G-75 column. Samples were run separately, immediately after isolation and although migration distances varied, mobility of the band on E was the same as the major band on gel C.

eluted as a single molecular species (M_r 25 000) from the Sephadex column run at 4°C and which showed one component (M_r 25 000) on SDS-polyacrylamide gel electrophoresis (Fig. 4). The unexpected finding of the larger molecular species on centrifugation suggested that aggregation was occurring, possibly promoted by the higher temperature at which centrifugation was conducted. In one experiment purified enzyme was chromatographed on Sephadex at 20°C rather than at 4°C. Under these conditions two peaks of activity were eluted, one at an approx. molecular weight of 50 000 accounted for 20% of the recovered activity and the other at 25 000 contained the remaining enzyme. This result supported the view that the enzyme tended to aggregate at room temperature. Extensive investigation of this behaviour was hindered by the low amounts of protein available and by its lability, so that a more detailed study was not undertaken.

Samples of the purified enzyme were subjected to isoelectric focusing in polyacrylamide gels. Gels were stained for protein with Coomassie brilliant blue R and one band was observed to have migrated into the gel with an estimated p*I* value of 5.9 (Fig. 5). However, some material remained at the origins of gels when concentrated samples were electrophoresed (Fig. 5B); this might indicate heterogeneity of the preparation. The staining at the origin was pronounced and that of the migrating band considerably diminished when samples were dialysed against water and then concentrated by freeze-drying prior to electrophoresis. This effect was less marked, though not obviated, when samples were concentrated by ultrafiltration and the intensities of staining of the two bands seemed to vary in a reciprocal manner, depending upon the method of concentrating samples and upon the degree of concentration effected; samples run without prior concentration showed little or no material at the origin (Fig. 5A). Thus, it seems likely that material not entering the gel was aggregated enzyme.

Characterization

The effect of a number of inhibitors on the hydrolysis of Np-Boc-Ala by the purified Np-Boc-Ala esterase was examined (Table III). Soya bean trypsin inhibitor was effective at an inhibitor/enzyme ratio greater than 5 : 1, causing greater than 90% inhibition. Phenylmethylsulphonyl fluoride at a concentration of 1 mM effectively inhibited (greater than 90%) both Np-Boc-Ala and azo-albumin hydrolysing activity. EDTA and $CaCl_2$ at concentrations of 5—20 mM had little effect on activity against either substrate. NaCl had little effect on activity between 0.15 and 0.5 M, unlike the activating effect found with the partially-purified enzyme. Removal of NaCl caused precipitation of the enzyme. $MgCl_2$ (20 mM), $ZnSO_4$ (1 mM) and heparin (50 units/ml) had no

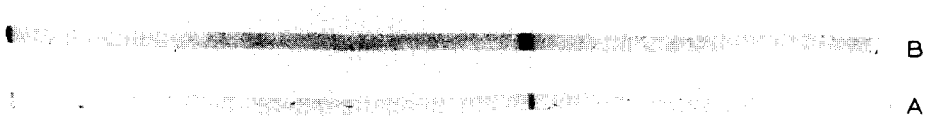

Fig. 5. Isoelectric focusing gels were run as described under Materials and Methods and stained for protein with Coomassie brilliant blue R. (A) 5 μg of proteinase, (B) 25 μg of proteinase.

TABLE III

EFFECTS OF INHIBITORS ON ENZYME ACTIVITY AGAINST Np-Boc-Ala

Enzyme activity is expressed as a percentage of the activity in the absence of inhibitors. EDTA, $CaCl_2$ and soya bean trypsin inhibitor were preincubated with enzyme for 5 min, phenylmethylsulfonyl fluoride (PMSF) was preincubated for 16 h at 4°C.

Compound	Final concentration	Activity (% of control)
PMSF	1 mM	0
EDTA	5 mM	100
	10 mM	100
$CaCl_2$	10 mM	100
	20 mM	100
Soya bean trypsin inhibitor	0.5 mg/ml	10
	0.25 mg/ml	10
	0.1 mg/ml	15
	0.01 mg/ml	90

effect on activity but rabbit serum (1 in 20 dilution) inhibited activity by 85%.

When the 1 M NaCl membrane extract or purified enzyme were incubated with elastin-orcein at different pH values (pH 5—11) no elastin breakdown was detected. Increasing the concentration of the membrane extract in the assay mixture from 50 to 400 μg, or of the purified enzyme from 20 to 100 μg, did not stimulate any elastolytic activity at any NaCl concentration at pH 8.6, even when incubation periods were extended up to 30 h. This was a surprising result, since enzyme in the same fraction hydrolysed Np-Boc-Ala and might, therefore, have been expected to hydrolyse elastin. It seemed possible that the enzyme could be present in the membrane extract as a proelastase and this was investigated by incubating the membrane extract with 25 μg trypsin in 0.01 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl at 37°C for a period of 30 min; under these conditions trypsin has been shown to stimulate elastase activity in extracts from bovine pancreas [20]. This preincubation did not promote any activity against elastin-orcein. Hog pancreatic elastase rapidly degraded elastin-orcein under the assay conditions employed.

In contrast to these results the membrane extract and the purified enzyme catalysed the hydrolysis of bovine tendon collagen and renal basement membrane as judged by the release of hydroxyproline-containing peptides from the insoluble substrate at pH 7.4. With both substrates activity was inhibited by soya bean trypsin inhibitor and phenylmethylsulphonyl fluoride. The purified enzyme was without activity against *N*-benzoyl-D,L-tyrosine ethyl ester and *N*-benzoyl-D,L-phenylalanine-2-naphthol ester.

A Lineweaver-Burk plot was determined using Np-Boc-Ala as the substrate; the graph was linear and the K_m was found to be $2.2 \cdot 10^{-4}$ M.

Freeze-drying the purified Np-Boc-Ala esterase resulted in losses of activity of up to 30%, difficulty was also experienced when trying to resolubilise the freeze-dried sample. Thus, as a means of concentration and preservation, freeze-drying was unsatisfactory. Storage of the enzyme in buffer, pH 7.4, at 4°C resulted in loss of enzyme activity, 10—15% within 24 h. However, in the presence of albumin the rate of inactivation was reduced considerably. In view of

TABLE IV

AMINO ACID COMPOSITION OF THE Np-Boc-Ala ESTERASE FROM THE GRANULES OF RABBIT LEUCOCYTES

Values are expressed as μg/mg protein and as residues/1000 residues.

Amino acid	μg/mg Protein	Residues/1000	Amino acid	μg/mg Protein	Residues/1000
Asp	120.0	117	Met	13.7	11
Thr	48.0	47	Ile	32.7	29
Ser	89.0	94	Leu	65.5	59
Glu	184.0	147	Tyr	42.0	27
Pro	54.5	55	Phe	33.0	24
Gly	100.0	152	His	24.2	19
Ala	60.5	82	Lys	59.8	49
Val	46.8	47	Arg	60.5	41

this data it is presumed that the loss of activity was due to self-digestion. At 60°C the esterase activity was rapidly destroyed with 33% and 5% of activity remaining after 6 and 15 min, respectively.

Table IV shows the amino acid composition determined for the purified enzyme.

Discussion

These experiments show that the cytoplasmic granules of rabbit polymorphonuclear leucocytes contain an enzyme which hydrolyses Np-Boc-Ala and which appears to be membrane-bound, requiring both detergents and a high salt concentration for solubilization. The enzyme could be purified by gel exclusion chromatography and resembled the elastase isolated from human spleen and leucocytes [21,2] in that it hydrolysed Np-Boc-Ala and was inhibited by phenylmethylsulphonyl fluoride and soya bean trypsin inhibitor. A major difference between the two enzymes is that no elastolytic activity could be detected with the rabbit enzyme. This total lack of elastolytic activity is supported by an earlier observation of Janoff et al. [22] who demonstrated Np-Boc-Ala esterase activity in rabbit leucocytes, but who also failed to detect any elastolytic activity. Since the enzyme was able to degrade macromolecular substrates, azo-albumin, collagen and basement membrane, the lack of elastolytic activity cannot be explained by assuming that the enzyme is a proelastase or that it remains associated with an inhibitor which prevents interaction with macromolecules, while permitting interaction with substrates of low molecular weight. We therefore must conclude that the proteinase is not an elastase although it catalyses the hydrolysis of Np-Boc-Ala, a substrate conventionally used for elastase. Consequently, assumptions that this substrate is specific for elastase must now be questionable.

It is of interest to note that there is no counterpart to the rabbit enzyme in human leucocytes. The rabbit enzyme does not hydrolyse substrates cleaved by Cathepsin G, and its properties, molecular size, isoelectric point and amino acid composition, are very different from those of human granulocyte elastase. Finally it is a serine proteinase and though active against collagen, it differs from

human collagenases which require metal ions for activity (see Ref. 1).

A high salt concentration was found to stimulate activity of the crude membrane extract 3-fold, but this effect was not so pronounced with the purified enzyme. It may be the case that the enzyme interacts electrostatically with other components in the preparation; this association would be prevented in the strong salt solution and the extent of any such association would be reduced during purification as other components were removed. Other proteinases have been reported which are activated similarly by high salt concentrations at neutral pH, these include enzymes from mast cells [23] and rabbit skin [24].

Although the purified enzyme showed a single band on polyacrylamide/SDS electrophoresis and isoelectric focusing gel electrophoresis, on centrifugation at 21°C several components were observed at molecular weights which were multiples of the size indicated by electrophoresis. These findings were unexpected since these high molecular weight components were present in samples twice-chromatographed by gel-exclusion, giving retention values consistent with a molecular weight close to 25 000. This suggests that the purified enzyme formed oligomers on centrifugation, which was conducted at 21°C rather than at 4° C, the temperature at which Sephadex columns were run. The formation of oligomers at higher temperatures has been shown to occur in the case of D-amino acid oxidase [25], and the binding of DNA to histone also occurs more favourably at higher temperatures [26]. No completely satisfactory explanation is available for such temperature-dependent associations; however, if hydrophobic interactions occur between monomers in solution then elevated temperatures will favour such associations [27].

The physiological function of the proteinase is unclear; however, since it is localised in the leucocyte granules it may be involved in the digestion of phagocytosed material. It is not known whether this enzyme can be released to the extracellular environment, as are proteases from human polymorphs [28], or whether it remains attached to the plasma membrane at the point of exocytosis as does alkaline phosphatase, an enzyme which is bound to the granule membrane [29]. Such localisation could provide a mechanism for limiting the effects of a potentially injurious enzyme. Additionally, close association between the leucocyte plasma membrane, possibly carrying the proteinase, and the surface of connective tissue elements could prevent access of plasma proteinase inhibitors to membrane-bound enzymes allowing them to express their activity.

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