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PURIFICATION OF GRANULOCYTE NEUTRAL PROTEASE FROM HUMAN BLOOD AND RHEUMATOID SYNOVIAL FLUID

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Summary

The neutral protease activity of human synovial fluid cells, like that of peripheral blood leucocytes, is located in a granule fraction. It can be solubilised by various agents but only 1 M neutral salts do so without inactivation. Salt-solubilised neutral protease has been purified (300×) from synovial fluid cells; like preparations obtained in the same way (600 × purified) from peripheral blood leucocytes, it has a broad pH profile of activity (pH 7—10.5) and in this, as well as in substrate specificity and sensitivity to activators and inhibitors, it behaves as a serine-histidine type protease similar to elastase (EC 3.4.21.11). The product showed two major components on polyacrylamide gel electrophoresis. Collagenase or chymotrypsin-like activity were not detected.

Introduction

Cells present in the synovial fluids of patients with rheumatoid arthritis contain neutral protease activity which can degrade the protein component of purified cartilage proteoglycans [1,2]. It can also cause loss of staining and release hexuronate from slices of articular cartilage and might contribute to the degradation of cartilage in rheumatoid arthritis [2]. More than 80% of the neutral protease activity of synovial fluid cell sonicates may be concentrated in a fraction (fraction P of ref. 2) which is insoluble in 0.1 M Tris-HCl buffer, pH 7.5, but soluble in buffer containing 1 M KCl. The neutral protease activity of fraction P probably derives from the granulocytes in synovial fluids and is similar in solubility, substrate specificity and susceptibility to various inhibitors

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and activators, to elastase-like neutral proteases found by others in granulocytes from various sources including human blood [3—13]. In this report we have addressed ourselves to the questions: (1) Is the insolubility of neutral protease in sonicates of granulocytes due to its location in subcellular granules or an artefact arising from association of the enzyme with other intracellular components? (2) To what extent can conventional protein purification techniques be used to purify the enzyme in spite of its peculiar solubility properties? Parallel experiments have been carried out with material from peripheral blood leucocytes and from synovial fluid cells. A brief account of part of this work has been published elsewhere [14].

Materials and Methods

Isolation of cells and subcellular fractions

Cells were separated from synovial fluids (10—50 ml containing $1 \cdot 10^4$—$5 \cdot 10^4$ cells per mm^3) of rheumatoid patients by centrifuging and washed with 0.15 M NaCl. In some experiments the washed cells were subjected to subcellular fractionation (see below). In others they were suspended in 0.1 M Tris-HCl buffer, pH 7.5 (4 ml), disintegrated by sonication, (MSE Ultrasonic Disintegrator, 2 min, 0—4°C) and cell debris removed by centrifuging (2000 × *g*, 15 min, 4°C). The insoluble fraction P was separated from the soluble fraction S (ref. 2) by centrifuging the opalescent supernatant at 100 000 × *g* for 1 h at 4°C (Spinco model L centrifuge, rotor no. 40E).

Peripheral blood was collected from normal humans, patients suffering from polycythaemia or haemochromatosis, or polycythaemic dogs, using acid/citrate/dextrose anticoagulant in standard transfusion packs. Leucocytes were isolated by the dextran sedimentation procedure of Janoff and Scherer [15]. Blood (400—900 ml) was mixed with two volumes Dextran T500 (Pharmacia), 3%(w/v) in 0.15 M NaCl and allowed to sediment in a siliconised 2-l glass measuring cylinder for 1 h at 4°C. The supernatant fraction was centrifuged (500 × *g*, 15 min) and the resulting pellet suspended in 25 ml 0.034 M NaCl. The suspension was shaken for 1 min and 25 ml 0.274 M NaCl added to restore isotonicity. The suspension was centrifuged (500 × *g*, 15 min) and the pellet once more exposed to hypotonic conditions to complete lysis of erythrocytes. The final leucocyte pellet contained 65—80% granulocytes which were not purified further.

Washed synovial fluid cells and peripheral blood leucocytes were suspended in 0.34 M sucrose at 4°C and disrupted by mild mechanical homogenisation as described by Weissmann et al. [16]. This resulted in 50—70% disruption of the cells and the homogenate was then separated into nuclear (plus intact cells), granule and cytosol fractions by differential centrifugation [16]. The particulate fractions, together with portions of unfractionated cells, were suspended in 0.1 M Tris-HCl buffer, pH 7.5, and all fractions subjected to sonication [2].

Solubilisation and purification of neutral protease

To investigate the solubilisation of particle-bound neutral protease, samples of fraction P from synovial fluid cells or of the granule fraction from

human blood leucocytes were dispersed in 4 ml of solutions of various solubilising agents in buffer (0.1 M Tris-HCl, pH 7.5, 0.1 M acetate, pH 5.0, or 0.1 M phosphate, pH 6.0) by sonication (MSE Ultrasonic Disintegrator, 2 min, 4°C) allowed to stand at 4°C for 1 h and centrifuged at 100 000 × *g* for 1 h at 4°C (Spinco Model L centrifuge, rotor no. 40E). Supernatant and pellet were separated and the latter dispersed in 0.1 M Tris-HCl buffer, pH 7.5. Neutral protease was assayed in both fractions using the hide powder azure method (see below).

To purify the neutral protease, preparations of granule fraction from peripheral blood leucocytes, or pooled fraction P from five or more samples of synovial fluid cells, were sonicated in 0.4 M KCNS, pH 7.5, to give a dispersion containing 100—200 mg protein per ml. After a small amount of undispersed material had been removed by centrifuging (10 000 × *g*, 20 min) the suspension was dialysed against 25% saturated $(NH_4)_2SO_4$ at 4°C for 16 h. The precipitate was removed by centrifuging (38 000 × *g*, 30 min) and solid $(NH_4)_2SO_4$ added to the solution, to 65% of saturation at 4°C. The precipitate, separated by centrifuging (38 000 × *g*, 30 min) was dissolved in 5 ml 0.1 M phosphate buffer, pH 6.0, containing 1.0 M KCl and the solution (approx. 50 mg protein per ml) subjected to gel-filtration chromatography on Sephadex G-75 (2.5 × 95 cm column, 25 ml/h, 4-ml fractions) at 4°C. The active fractions were pooled, concentrated by dialysis against solid Dextran T500 and dialysed against 0.1 M phosphate/acetate buffer, pH 4.2, containing 0.1 M NaCl. The solution (4—8 mg protein per ml) was applied to a column (0.9 × 15 cm) of sulphopropyl Sephadex C25 which was then washed with 6 ml of the same buffer. The column was then eluted with a linear NaCl gradient (starting eluent, 50 ml 0.1 M phosphate/acetate buffer, pH 4.2, containing 0.1 M NaCl; limiting eluent, 50 ml of the same buffer, containing 0.8 M NaCl). Active fractions were pooled, concentrated and dialysed against 0.05 M phosphate buffer, pH 5.0.

Enzyme assays

Proteoglycan-degrading activity of enzyme preparations was determined by measuring the reduction of the viscosity of solutions of purified bovine nasal septum cartilage proteoglycan as described previously [1]. One unit of activity is that amount of enzyme which causes 20% decrease in specific viscosity in 10 min when added, in 0.2 ml, to 2 ml of substrate solution (0.4% in 0.1 M Tris-HCl buffer, pH 7.5) at 25°C. Neutral protease activity was determined using hide-powder azure (grade B, Calbiochem Ltd, London) as substrate. One unit of protease activity is that amount of enzyme which dissolves 1 mg substrate in 1 h under standard conditions [2]. The assay medium contained 0.2 M KCl at which concentration the crude neutral protease of synovial fluid cells (fraction P) shows 75% of its maximal activity with this insoluble substrate [2]. Elastase activity was determined using elastin covalently dyed with Remazol Brilliant Blue R as substrate, in the presence of 0.2 M KCl [2]. The hydrolysis of *N*-(*t*-Boc)-L-alanine *p*-nitrophenyl ester (Sigma, London) and *N*-acetyl-L-alanyl-L-alanyl-L-alanine methyl ester (Sigma, London), two synthetic substrates for elastase (EC 3.4.21.11), was followed at 37°C by the methods of Janoff [9,17].

In order to investigate the collagenase activity of purified neutral protease, the effect of the enzyme (approx. 50 protease units per ml) on the viscosity of a solution of calf-skin neutral salt-soluble collagen (0.1% in 0.1 M Tris · HCl buffer, pH 7.5, containing 0.4 M NaCl) at 25°C was determined as previously described [1]. For comparison the same experiment was also done with trypsin (approx. 10 μg/ml). In addition diisopropylphosphofluoridate (Sigma, London) was added (final concentration 10^{-4} M) to a sample of the treated collagen (16 h) to inhibit the protease; the collagen was then denatured with 7 M urea and subjected to electrophoresis on polyacrylamide gels [18]. At the same time trypsin-treated collagen and untreated collagen were also subjected to electrophoresis. The gels were stained with Amido Black.

β-Glucuronidase was assayed as described by Fishman [19] using phenolphthalein-β-D-glucuronide (Koch-Light Ltd, Colnbrook, Bucks), as substrate. Protein concentration was determined by the method of Miller [10] using bovine serum albumin as a standard or, when solutions were too dilute for this method, the direct spectrophotometric methods of Layne [21] or Murphy and Kies [22].

Polyacrylamide gel electrophoresis

Purified enzyme was subjected to electrophoresis on cylindrical polyacrylamide gels as described by Davis [23]. The running gel (50 mm long, 1 ml) contained 7% acrylamide (B.D.H. Ltd, Poole, Dorset) 0.25% methylene bisacrylamide (Koch-Light) in 0.025 M Tris-HCl buffer, pH 7.5. The spacer gel (5 mm) contained 5% acrylamide and 0.15% methylene bisacrylamide in the same buffer. The gels were pre-electrophoresed at 200 V for 2 h using 0.035 M β-alanine/acetate buffer, pH 4.5 in both electrode vessels. The sample (50 μl, approx. 5 protease units) containing 0.584 M sucrose was then layered on the spacer gels and electrophoresis carried out at 1 mA per tube for 90 min. Gels were either stained for protein with Amido Black (1% in 7.5% acetic acid) or cut into 1.7-mm thick slices. Each slice was homogenised in 1.4 ml 0.1 M Tris-HCl buffer, pH 7.5, containing 0.2 M KCl and 0.05% Triton X-100, and assayed for neutral protease using hide powder azure as substrate.

Chondroitin sulphate and heparin were obtained from Sigma U.K., London, and dextran sulphate from Pharmacia.

Results and discussion

The subcellular fractionation studies show (Table I) that the neutral protease activity of both synovial fluid cells and peripheral blood leucocytes occurred predominantly in the granule fraction. This was particularly apparent in the blood leucocytes. With the synovial fluid cells the samples of material were so small that the method of disruption of the cells appears to have been less efficient, as indicated by the greater concentration of protease in the nuclear fraction, which undoubtedly contained substantial numbers of unbroken cells. This is supported by the relatively high proportion of β-glucuronidase which was also recovered in the nuclear fraction. In both synovial fluid cells and blood leucocytes a significant proportion of the protease activity was recovered in the cytosol fraction. These results are thus consistent with our earlier finding

TABLE I

DISTRIBUTION OF ENZYME ACTIVITY IN SUBCELLULAR FRACTIONS OF BLOOD LEUCOCYTES AND SYNOVIAL FLUID CELLS

Figures in parentheses are the numbers of samples. All data are ± standard deviation. With human blood leucocytes, neutral protease was assayed using hide powder azure as substrate. With synovial fluid cells purified cartilage proteoglycan was the substrate. As shown previously [2] there is a good linear relationship between the two assays.

Cells	Enzyme	Subcellular fraction	Percent of total enzyme activity	Specific activity of fraction / Specific activity of homogenate
Human blood leucocytes	Neutral protease (8)	N	15 ± 8	0.78 ± 0.07
		G	76 ± 9	3.19 ± 0.40
		C	6.7 ± 3.3	0.13 ± 0.01
	β-Glucuronidase (8)	N	13.0 ± 4.4	0.86 ± 0.09
		G	50 ± 2	2.14 ± 0.13
		C	37 ± 6	0.63 ± 0.05
Synovial fluid cells	Neutral protease (10)	N	38 ± 13	1.14 ± 0.02
		G	50 ± 7	4.30 ± 0.31
		C	14.5 ± 6	0.42 ± 0.06
	β-Glucuronidase (6)	N	38 ± 7	1.03 ± 0.07
		G	20.5 ± 8	1.54 ± 0.13
		C	42.0 ± 7	1.06 ± 0.21

[2] that 80% of the neutral protease activity of a crude extract of synovial fluid cells which had been disrupted by sonication in 0.1 M Tris · HCl buffer, pH 7.5, sedimented at 100 000 × *g* in 1 h at 4°C (fraction P of ref. 2) leaving 20% of the protease activity in solution (fraction S of ref. 2).

When fraction P from synovial fluid cells or the granule fraction of human blood leucocytes were extracted with Triton X-100, Lubrol W or sodium deoxycholate (0.2%, pH 7.5) less than 8% of the neutral protease was solubilised. A number of potential solubilising agents caused some solubilisation of neutral protease from fraction P (Table II); results with granule fraction were similar. All these compounds caused considerable inactivation however. In agreement with earlier observations on fraction P (ref. 2) more than 90% of the neutral protease of granule fraction was solubilised by 1 M KCl at pH 7.5 with negligible inactivation. Similar results were obtained with 1 M $(NH_4)_2SO_4$ and 1 M KCNS at pH 7.5 and with 1 M KCl at pH 6.0. At pH 5.0 however, 1 M KCl

TABLE II

SOLUBILISATION OF NEUTRAL PROTEASE FROM FRACTION P OF SYNOVIAL FLUID CELLS

All extractions were at pH 7.5, for details see text. The concentration of protease in the dispersions of fraction P in the solubilisation media was 200—300 units per ml.

Agent		Activity recovered in supernatant (%)	Inactivation (%)
Sodium dodecyl sulphate	0.2%	58	70 (after 6 h)
	0.5%	5	90
	Urea 3 M	9	56
Heparin	0.5%	62	30
Chondroitin sulphate	0.5%	32	26
Dextran sulphate	1.0%	15	55

solubilised only 65% of the neutral protease. It is concluded that the insolubility of the neutral protease previously observed in fraction P is not an artefact resulting from association of the enzyme with components of the cytosol during preparations but is due to binding of the enzyme to components of the granule fraction. Moreover, this interaction appears to be primarily ionic in nature.

Gel chromatography of the blood leucocyte granule neutral protease (eight samples) in the presence of sufficient salt to solubilise it yielded a single peak of activity (Fig. 1). Synovial fluid cell neutral protease behaved in a similar manner. Chromatography on Sephadex G-150 also gave a single peak of neutral protease activity. The activity in pooled active fractions was insoluble (i.e. sedimented at 100 000 × *g* in 1 h) after dialysis against 0.1 M Tris-HCl buffer at pH 7.5. Chromatography on sulphopropyl Sephadex (Fig. 2) also gave a single peak of activity with all samples studied. The pooled active fractions were now soluble in 0.1 M Tris-HCl buffer at pH 7.5. They were routinely stored in solution in 0.05 M phosphate buffer, pH 5.0, at −20°C without loss of activity up to 6 months. Table III shows typical data for purification and recovery. For all human samples recovery ranged from 10 to 30% and purification from 300- to 600-fold. With dog leucocytes recoveries (25—45%) and purification (500—800-fold) were somewhat higher. Proteoglycan-degrading activity was recovered with overall purification approximately equal to that of neutral protease activity.

When the purified neutral protease was subjected to analytical gel electrophoresis two major protein brands migrated towards the cathode with a mobility similar to that of bovine trypsin. Most of the neutral protease activity was associated with these bands (Fig. 3). Two slower minor protein bands were also

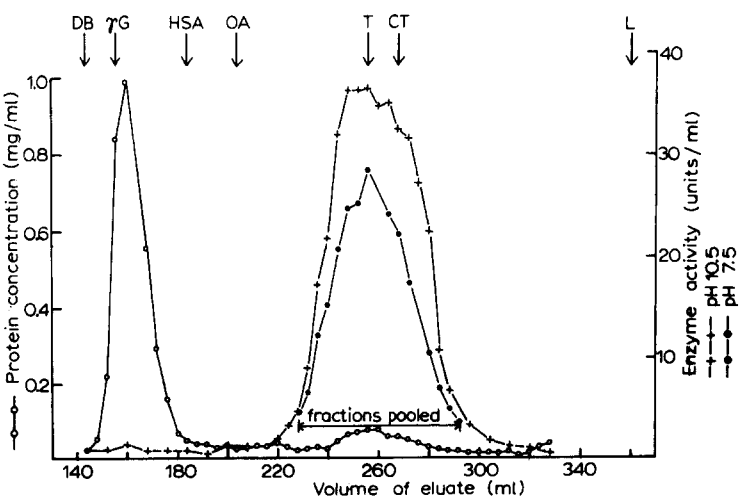

Fig. 1. Chromatography of neutral protease from human blood leucocyte granule fraction on Sephadex G-75. Protease activity of fractions was determined at pH 7.5 (●———●) and at pH 10.5 (+———+). The protein concentration of fractions was estimated from their absorbance at 280 nm. Arrows indicate the elution volumes of Dextran Blue (DB), γ-globulin (γG), human serum albumin (HSA), ovalbumin (OA), trypsin (T), chymotrypsin (CT) and lysozyme (L).

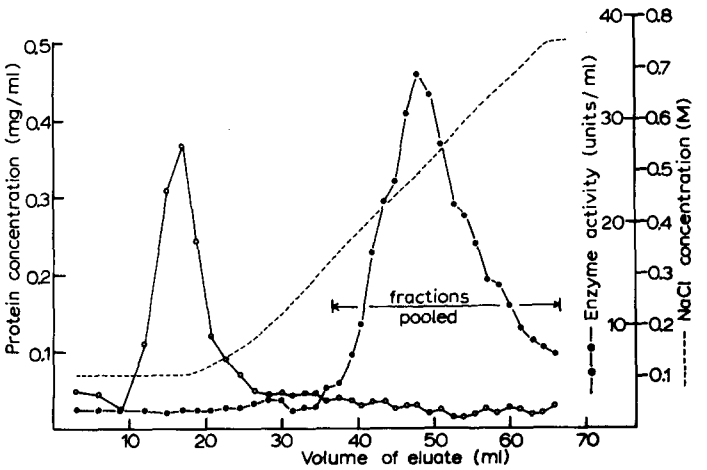

Fig. 2. Chromatography of neutral protease from human blood leucocyte granule fraction on sulphopropyl Sephadex G-25. Protease activity (●———●) was determined at pH 7.5 and protein concentration (○———○) estimated from absorbance at 280 nm.

observed, the faster of which was associated with some protease activity. Insufficient material was available to exploit this separation on a preparative scale and further studies were carried out with the active fractions from sulphopropyl Sephadex chromatography.

The purified enzyme from both synovial fluid cells and blood leucocytes had the same substrate specificity as the crude neutral protease (fraction P) from synovial fluid cells [2]. In particular it hydrolysed the elastase substrates Remazol Brilliant Blue-elastin, Cbz-L-alanine *p*-nitrophenyl ester, *N*-(*t*-Boc-L-alanine *p*-nitrophenyl ester and *N*-acetyl-L-alanyl-L-alanyl-L-alanine methyl

TABLE III

PURIFICATION OF NEUTRAL PROTEASE FROM HUMAN LEUCOCYTES AND SYNOVIAL FLUID CELLS

Neutral protease was assayed by the hide powder azure procedure, see text.

	Leucocytes			Synovial fluid cells		
	Total protease (units)	Specific activity (units/mg protein)	Purification	Total protease (units)	Specific activity (units/mg protein)	Purification
Unfractionated cell homogenate (sonicated)	7440	7.9	1.0	5521	4.9	1.0
Granule fraction (leucocytes) or fraction P (synovial fluid cells)	7980	28.6	3.6	4048	18.2	3.7
$(NH_4)_2SO_4$ fraction	4790	112	14	3370	65	13
Sephadex G-75	2922	1059	134	2224	394	80
SP Sephadex C25	1470	4742	599	1557	1497	306
Overall recovery (%)	20			28		

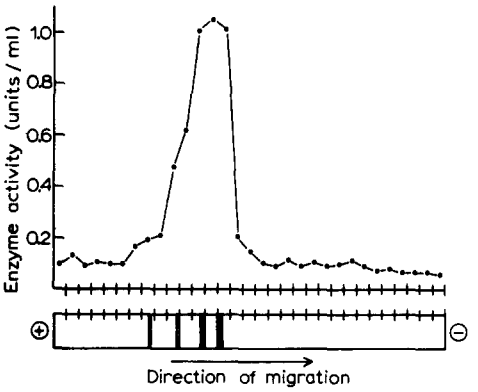

Fig. 3. Distribution of protein and neutral protease in polyacrylamide gels after electrophoresis of purified enzyme. The lower diagram as visualised with Amido Black and the upper diagram the concentration of protease (pH 7.5) in homogenates of gel slices.

ester. With the latter two synthetic substrates approximate values of K_m were $7.7 \cdot 10^{-4}$ and $2.1 \cdot 10^{-4}$ mol/l, respectively.

Purified enzyme preparations from synovial fluid also reduced the specific viscosity of a solution of neutral salt-soluble collagen by 20—25% in 16 h as did trypsin. Electrophoresis of enzyme-treated collagen, after it had been denatured, showed that the proportion of the collagen in the form of α chains was higher than in untreated collagen while the proportion in the form of β and γ cross-linked chains was lower. Altered α and β components, such as those observed in collagen after collagenase treatment, were not observed. These results indicate that the neutral protease attacks the extra-helical parts of the collagen molecule but does not contain true collagenase activity [26].

The crude neutral protease (fraction P) from synovial fluid cells had a broad pH profile of activity, with several protein substrates [1,2], extending

TABLE IV

EFFECT OF INHIBITORS AND ACTIVATORS ON NEUTRAL PROTEASE FROM SYNOVIAL FLUID CELLS

The concentration of enzyme was approx. 1 protease unit per ml.

	Change of activity (%)	
	Purified enzyme	Fraction P
Soybean trypsin inhibitor (50 μg/ml)	−73	−100
Heparin (50 units/ml)	−42	−68
Aprotinin (Trasylol) (100 units/ml)	−30	−76
Diisopropylphosphorofluoridate (10^{-4} M)	−100	−96
ε-Aminohexanoic acid (25 mM)	−4	−27
Iodoacetamide (25 mM)	−9	−36
Cysteine (25 mM)	+5	+21
EDTA (25 mM)	−97	−29
Ca^{2+} (25 mM)	+21	+21
Mg^{2+} (25 mM)	+26	+21
Mn^{2+} (25 mM)	+27	+24

from pH 7 to 10.5. The pH vs activity curve for purified enzyme, with hide powder azure as substrate, was not significantly different from that previously observed with fraction P. In addition the ratio of activity at pH 10.5 to activity at pH 7.5 was approximately constant for all the active fractions resulting from chromatography on Sephadex G-75 (Fig. 1). There is thus no evidence of separation of proteases with markedly different pH optima and the purified enzyme has a broad pH vs activity curve similar to that of elastase (EC 3.4.21.11) [24].

A number of potential activators and inhibitors had qualitatively similar effects on the purified synovial fluid cell enzyme and on the crude enzyme of fraction P (Table IV). There were, however, some significant differences; thus iodoacetamide, cysteine and ϵ-aminohexanoic acid had very little effect on the purified enzyme while EDTA inhibited the purified enzyme to a greater extent than fraction P, suggesting that traces of contaminating proteolytic activity sensitive to these reagents has been removed during purification. *N*-α-*p*-tosyl-L-lysine chloromethyl ketone and L-1-tosylamido-2-phenylethyl-chloromethyl ketone did not affect the activity of the purified enzyme at the low concentration (0.5 mM) at which they specifically inhibit trypsin and chymotrypsin, respectively [27,28]. At higher concentration (15 mM) there was considerable (approx. 80%) inhibition of the protease by both compounds. The general properties of the enzyme thus indicate that is is a serine-histidine type protease having a fairly broad amino acid specificity, similar to elastase [24]. The elution volume of the protease on Sephadex G-75 (Fig. 2) is consistent with a molecular weight between 22 000 and 24 000.

Schmidt and Havemann [25] recently described the purification of the neutral proteases from a granule fraction of human peripheral blood granulocytes by a somewhat similar experimental procedure to ours. Our purified neutral protease resembles very closely their "elastase-like protease" but we obtained no evidence for the presence in our preparations of their "chymotrypsin-like" protease. We previously found, however, that fraction S of sonically disrupted synovial fluid cells, i.e. the fraction soluble in 0.1 M Tris · HCl buffer, pH 7.5, contains a complex mixture of proteolytic enzymes which account for some 20% of the neutral protease activity of the cells and include some which hydrolyse the chymotrypsin substrates acetyltyrosine ethyl ester and benzoyltyrosine ethyl ester [2]. After subcellular fractionation of both synovial fluid cells and human blood leucocytes some 10—15% of the neutral protease activity was removed in the cytosol fraction and it is possible that using our subcellular fractionation procedure any chymotrypsin-like protease is located in this fraction rather than in the granule fraction.

The electrophoretic mobility and heterogeneity of the purified neutral protease is similar to that reported by Sweetman et al. [13] and Ohlsson and Olssen [12] for elastase-like esterases from human leucocytes and by Schmidt and Havemann [25] for their elastase-like protease. The cationic nature of the neutral protease was confirmed in preliminary isoelectric focusing experiments [14] and it no doubt accounts for the solubilising action of polyanions the physiological significance of which has been discussed elsewhere [14].

Acknowledgements

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