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Isolation and Some Molecular Parameters of Elastase-Like Neutral Proteinases from Horse Blood Leucocytes

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Cytoplasmic granules were isolated from horse blood polymorphonuclear leucocytes by the heparin method and extracted with 0.9% NaCl by repeated freezing. Soluble proteins were separated on a column of Sephadex G-75 followed by chromatography on a column of CM-Sephadex with a NaCl gradient. Gel filtration, density-gradient centrifugation, isoelectric focusing and 0.1% sodium dodecyl sulphate/polyacrylamide-gel electrophoresis at pH 7.0 and at pH 4.5 were used to determine molecular parameters of proteinases. Three enzymes hydrolysing both casein and *N*-benzyloxycarbonyl-L-alanine nitrophenyl ester were found in the granule extract: proteinase 1, mol.wt. 38000, pI 5.3; proteinase 2A, mol.wt. 24500, pI 8.8; and proteinase 2B, mol.wt. 20500, pI above 10. The latter two elastase-like proteinases were purified to apparent homogeneity.

The cytoplasmic azurophil granules of human polymorphonuclear leucocytes contain several neutral proteinases active above pH 7 and presumably involved in the inflammatory reaction. The most prevalent are elastase-like enzymes showing broad proteolytic activity as well as alanine nitrophenyl esterase activity (Janoff, 1972, 1973; Folds *et al.*, 1972; Ohlsson & Olsson, 1974; Schmidt & Havemann, 1974; Rindler-Ludwig *et al.*, 1974; Dewald *et al.*, 1975).

As distinct from human leucocytes, the granules from rabbit polymorphonuclear leucocytes contain mainly acid proteinases and small amounts of a different neutral proteinase active against histones (Davies *et al.*, 1971). Lebez *et al.* (1972) reported that extracts from pig leucocytes hydrolyse some serum proteins both at pH 3.5 and at 8.0. The enzymes responsible have been purified by Kopitar & Lebez (1975). We demonstrated that horse blood leucocyte granules contain very active neutral proteinases that can be inhibited by serum α_1-antitrypsin and α_2-macroglobulin (Koj *et al.*, 1972). During incubation at pH 7–8 the granule extract digests casein more easily than haemoglobin or serum albumin and is activated by a high concentration of urea (Chudzik, 1972). Polyacrylamide-gel electrophoresis and gel filtration on columns of Sephadex G-50 indicated that the enzymes responsible for this activity are basic proteins of low molecular weight (Chudzik, 1972; Dubin *et al.*, 1974). In the present paper we report the procedure for purification of two esterase-like neutral proteinases from horse blood leucocytes and also describe their subcellular distribution and some molecular parameters.

Materials and Methods

Reagents

All reagents were of analytical grade unless otherwise stated. Heparin was obtained from Polfa, Warsaw, Poland; bovine serum albumin was from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.; casein, acrylamide and *NN*-methylenebisacrylamide were from BDH Chemicals Ltd., Poole, Dorset, U.K.; benzyloxycarbonyl-L-alanine 4-nitrophenyl ester (Z-Ala-ONp) was from Fluka, Lucerne, Switzerland; 2-hydroxy-5-nitrophenyl sulphate (potassium salt), ovalbumin (five times crystallized), α-chymotrypsin (thrice crystallized) and crystallized horse myoglobin were all from Koch–Light Ltd., Colnbrook, Bucks., U.K.; pepsin (thrice crystallized) and α-chymotrypsinogen A (6-times crystallized from bovine pancreas) were from Sigma Chemical Co., St. Louis, Mo., U.S.A.; bovine trypsin (twice crystallized) was from Schuchardt, München, West Germany; crystalline lysozyme from egg white, cytochrome *c* from horse heart and L-arginine were all from Reanal, Budapest, Hungary; Aquacide was from Calbiochem, Los Angeles, Calif., U.S.A.; Amido Black 10B was from E. Gurr Ltd., High Wycombe, Bucks., U.K.; Ampholine was from LKB, Stockholm, Sweden; Sephadex G-75, CM-Sephadex A-25 and Blue Dextran 2000 were from Pharmacia, Uppsala, Sweden; $Na^{131}I$ (carrier-free, 10mCi/ml) was from IBJ, Swierk, Poland; $Na^{125}I$ (carrier-free, 100mCi/ml) was from The Radiochemical Centre, Amersham, Bucks., U.K.; sodium dodecyl sulphate and Coomassie Brilliant Blue R-250 were from Serva, Heidelberg, West Germany.

Isolation of leucocytes and fractionation of subcellular components

Leucocytes were isolated from 9 litres of fresh citrated horse blood by free sedimentation of erythrocytes and centrifugation of the plasma at 600*g* for 15 min. Residual erythrocytes were removed by a brief exposure to 0.2% NaCl solution. The leucocyte pellet, containing 1×10^{10}–1×10^{11} cells and consisting of 80–85% of polymorphonuclear leucocytes, was suspended in a small volume of 0.2M-sucrose and transferred to 400ml of 0.2M-sucrose with 150 units of heparin/ml. The cells were disrupted by intensive shaking of this suspension, as suggested by Chodirker *et al.* (1968). The viscous solution was centrifuged at 2000*g* for 15 min to remove nuclei and cell debris. The granular fraction was then obtained by centrifuging at 20000*g* for 30 min. The granular pellet was washed in 0.3M-sucrose solution by centrifuging under the same conditions and finally suspended in 20ml of 0.9% NaCl solution. The suspension was frozen and thawed three times and then centrifuged at 20000*g* to yield a pale-green supernatant (extract I). The insoluble residue was suspended in 0.9% NaCl and treated with 4 vol. of cold (−5°C) acetone. After centrifugation the pellet was dried under decreased pressure over KOH pellets and extracted with 10ml of 0.9% NaCl (extract II). Combined extracts were used as the source of enzymes. The extracts were either stored at −20°C or were concentrated by precipitation with 4 vol. of cold acetone, dried over KOH in vacuum and kept in the form of powder in the refrigerator for several months without loss of proteolytic activity. The protein content of the subcellular fractions was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Determination of enzymic activities

Proteolytic activity was determined in the following way. A portion (0.4ml) of suitably diluted enzyme preparation (containing 10–100 μg of protein) was mixed with 0.3ml of 0.1M-sodium phosphate buffer, pH7.4, and 0.5ml of the substrate [a 5% (w/v) solution of casein in 0.1M-sodium phosphate buffer containing 5M-urea; final pH7.4]. The sample was incubated in a water bath at 37°C for 60 min. The reaction was stopped by the addition of 3ml of 10% (w/v) trichloroacetic acid. The protein precipitate was left for 30 min at room temperature (20°C) then filtered off and the E_{280} of the filtrate was determined. Blank samples contained all reagents but in this case the trichloroacetic acid was added immediately after substrate. For convenience it was assumed that 1 unit of proteolytic activity corresponds to a ΔE_{280} of 0.1 unit in samples incubated under the above conditions. Since the crude enzyme preparation contains nucleic acids that can be hydrolysed in the incubated samples (thus increasing extinction at 280nm), some control experiments were carried out with the independent measurement of protein-degradation products by the Lowry *et al.* (1951) method. It was found that increase in the absorbance at 280nm was always strictly correlated with the increase of protein-degradation products in samples incubated with casein.

Esterolytic activity was determined with a synthetic substrate of elastase, *N*-benzyloxycarbonyl-L-alanine 4-nitrophenyl ester, by the method of Janoff (1969) with the following modification: samples (0.05–0.2ml) of the enzyme were mixed with 3ml of substrate (0.2mM) in 0.05M-sodium phosphate buffer, pH6.5, containing 10% (v/v) ethanol (thus the final substrate concentration was between 0.187 and 0.197mM). The $\Delta E_{347.5}$ against an appropriate blank was recorded for 3 min at 25°C in a Unicam SP.700A spectrophotometer. It was assumed that 1 unit of esterolytic activity results in the formation of 1 μmol of *p*-nitrophenol/min under the above conditions.

The activity of arylsulphatase A (EC 3.1.6.1) was measured as described by Baum *et al.* (1959) by incubating 0.5ml of enzyme solution with 0.5ml of substrate (12 μmol of 2-hydroxy-5-nitrophenyl sulphate) in appropriate buffer for 30 min at 37°C. It was assumed that 1 unit of arylsulphatase A activity results in the formation of 1 μmol of *p*-nitrocatechol/h under the above conditions.

Sephadex gel filtration

Molecular sieving on analytical scale was performed on a column (1.4cm×100cm) of Sephadex G-75 previously equilibrated with 0.05M-sodium phosphate buffer, pH6.0, containing either 0.15M-NaCl, 1M-NaCl or 2M-KCl. The extract of leucocyte granules was dialysed overnight against appropriate buffer and a 0.3ml sample containing approximately 10mg of protein was applied to the column. The flow rate of 0.3ml/min was carefully controlled with a two-step pump and the E_{260} of the effluent was continuously recorded by means of the optical detection system of a JEOL amino acid analyser. Neutral proteinase was located in the effluent by means of enzymic activity, whereas the following components were used for column calibration: Blue Dextran 2000 (mol.wt. 45000), ovalbumin, pepsin (35000), trypsin (23000), α-chymotrypsinogen A (22000), α-chymotrypsin (21600), lysozyme (17500), myoglobin (17000) and cytochrome *c* (13000).

Molecular sieving on a preparative scale was carried out on a column (3.5cm×90cm) of Sephadex G-75 equilibrated with 0.05M-sodium phosphate buffer, pH6.0, containing 0.15M-NaCl. Leucocyte granules (approx. 150mg) were applied to the column and fractions (5ml) were collected at the flow rate of 20ml/h. The E_{280} (determined in individual fractions in a VSU2P spectrophotometer) was taken as a

measure of protein content; selected fractions were assayed for neutral proteinase and alanine nitrophenyl esterase activities, then pooled and concentrated with Aquacide.

Ion-exchange chromatography

Enzyme samples were applied to a column (2cm×20cm) of CM-Sephadex A-25 equilibrated with 0.05M-sodium phosphate buffer, pH 7.4. Elution was carried out with this buffer in a linear NaCl gradient (0–1M-NaCl) at the flow rate of 20ml/h. The E_{280}, proteinase activities and NaCl content (argentometric titration) of the effluent were determined. The most active fractions were pooled, concentrated and used for further analysis.

Polyacrylamide-gel electrophoresis

Electrophoresis was carried out in 7% (w/v) polyacrylamide gel in glass tubes (0.6cm×6cm) in β-alanine buffer, pH 4.5, as described by Reisfeld *et al.* (1962). Proteins were stained with Amido Black 10B. Proteolytic activity was determined as follows: the unstained gel was sliced into 3mm sections immediately after electrophoresis, each section being placed in a test tube with 0.5ml of 0.1M-phosphate buffer, pH 7.4. The tubes were left overnight at 4°C and then used directly for the determination of enzymic activity.

Isoelectric focusing

The full leucocyte-granule extract (approx. 15mg of protein), or the partly purified proteinase (3mg), were placed in a continuous sucrose gradient (5–20%, w/v) with 1% Ampholine in a 110ml column. The gradient was run for 48h (300V, 0.5mA) and fractions (2.5ml) were collected and used for the determination of pH, neutral proteinase and alanine nitrophenyl esterase activities.

Ultracentrifugation in sucrose gradients

Partly purified leucocyte proteinase 2 after Sephadex G-75 column chromatography was mixed with markers, ^{131}I-labelled α-chymotrypsinogen or ^{125}I-labelled myoglobin, labelled by the ICl method (McFarlane, 1964). A sample (0.2ml) of the mixture was applied to the top of linear sucrose gradient (5–20%, w/v) prepared as described by Britten & Roberts (1960) in a 5ml tube. Centrifugation was carried out in a 3×5ml swing-out bucket of a VAC-60 (Janetzki) preparative ultracentrifuge for 20h at 38000rev./min ($r_{av.}$ 5.5cm) at approx. 5°C. Then 0.1ml fractions were collected as described by Szybalski (1968) and were used for the determination of ^{131}I or ^{125}I radioactivities in a well-type scintillation counter (USB-2, Swierk) equipped with a one-channel amplitude analyser A-21 (counting efficiency was 35% for ^{131}I and 28% for ^{125}I). The samples were then used for measuring the activities of proteinase or alanine nitrophenyl esterase. It was found that trace amounts of ^{131}I-labelled chymotrypsinogen used in the experiment did not give any detectable proteolytic activity. The results obtained were used for the calculation of the sedimentation coefficient of leucocyte proteinase by the method of Martin & Ames (1961).

Molecular-weight determinations by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

The enzyme purified on columns of Sephadex G-75 and CM-Sephadex was subjected to electrophoresis in 7% (w/v) polyacrylamide gel in 0.1M-sodium phosphate buffer, pH 7.0, with 0.1% sodium dodecyl sulphate as described by Weber & Osborn (1969). Cytochrome *c* (mol.wt. 13000), ovalbumin (45000) and bovine serum albumin (68000) were used as reference proteins. Proteins were stained with 0.25% Coomassie Brilliant Blue and relative mobilities were then calculated.

Results

Table 1 presents the results of a typical experiment with subcellular fractionation of horse leucocytes. Arylsulphatase A was used as the enzymic marker of the granular fraction and, as expected, over 60% of the enzyme activity was found in the granule extract. The subcellular distribution of neutral proteinase is rather similar to arylsulphatase, but the calculated recovery reaches a paradoxical value of 150%. This can be explained by the latency of the enzyme and its tight association with granule components, or by the presence of proteinase inhibitors in the cell sap. The existence of such inhibitors was described in human leucocytes by Janoff & Blondin (1971), and in rabbit leucocytes by Davies *et al.* (1971). In agreement with the observations of the above-mentioned authors, the proteolytic activity of the full lysate of horse leucocytes was significantly increased by the addition of heparin or 2M-KCl. On the other hand, these compounds had only a slight effect on the proteolytic activity of leucocyte-granule extract, and heparin even inhibited the enzyme by approximately 10%. Further observations indicated that neutral proteinase could be precipitated from the leucocyte-granule extract at the heparin/protein ratio of 50i.u./mg. The precipitate was readily solubilized in 1M-NaCl or by the addition of 5mg of arginine/mg of protein. During this procedure a three- to five-fold increase in the specific activity of proteinase was observed, but the method was not suitable for enzyme purification because of the difficulty of removing heparin.

A significant purification of neutral proteinase was obtained during filtration of the granule extract on a column of Sephadex G-75 (Fig. 1). Taking into account the absorbance at 280nm the extract was

Table 1. *Distribution of protein, arylsulphatase A and neutral proteinase in subcellular fractions of horse leucocytes*

Protein content is expressed as mg/fraction; arylsulphatase A and proteinase activities are in units/fraction. The proteinase activity in leucocyte lysate was determined in the presence of heparin (without heparin 1800 units of proteinase in cell lysate was found). Extract I was obtained by repeated freezing and thawing of the granule suspension in 0.9% NaCl, and extract II was obtained after dehydration in cold acetone of the insoluble residue from the first extraction.

		Leucocyte lysate	Nuclear fraction	Final supernatant	Sucrose washings	Leucocyte granules: Extract I	Leucocyte granules: Extract II	Recovery
Protein	(mg)	3620	92	3240	56	197	128	3713
	(%)	100	2.5	89.5	1.5	5.4	3.5	102.4
Arylsulphatase A	(units)	440.6	13.4	118.0	8.7	210.0	83.2	433.3
	(%)	100	3.0	26.8	2.0	47.6	18.9	98.3
Neutral proteinase	(units)	6400	120	900	50	6230	2255	9555
	(%)	100	1.9	14.0	0.8	97.3	35.2	149.3

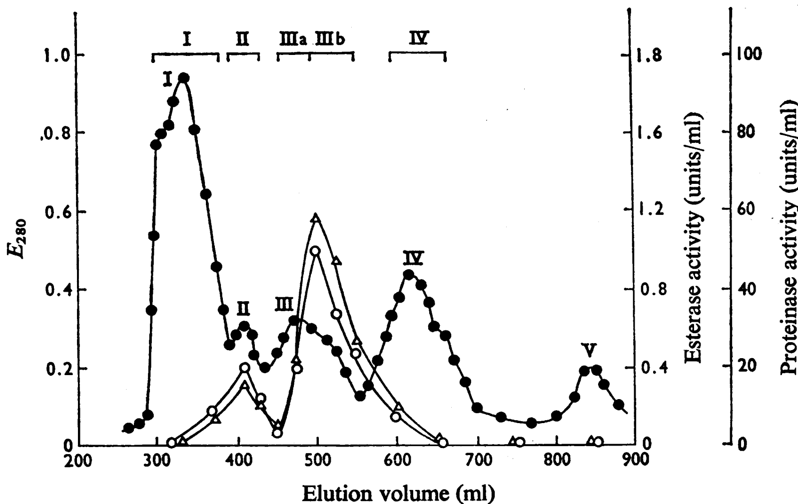
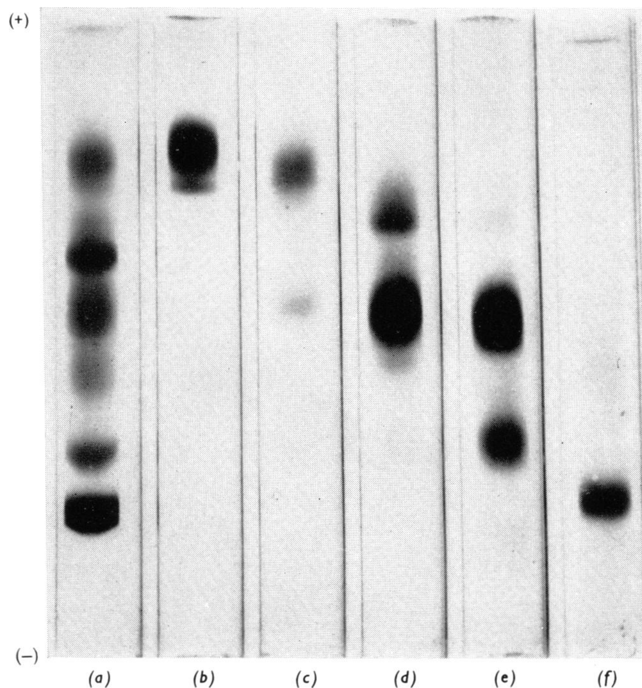


Fig. 1. *Sephadex G-75 chromatography of leucocyte-granule extract*

About 150mg of protein in 3ml were applied to a column (3cm×90cm) of Sephadex G-75 equilibrated with 0.05M-sodium phosphate buffer, pH6.0, containing 0.15M-NaCl. The column was eluted with this buffer and 5ml fractions were collected. ●, E_{280}; △, caseinolytic activity; ○, Z-Ala-ONp esterase activity. Enzyme units are as defined in the Materials and Methods section. For details of peaks I–V, see the text. Pooled fractions subjected to electrophoretic analysis (cf. Plate 1) are shown on the top of the elution diagram.

separated into five distinct peaks I–V, with two separate maxima of enzymic activity (Fig. 1). The first one, coinciding with the protein peak II, always constituted a minor component and in some leucocyte extracts was present in trace amounts only. The main proteolytic and esterolytic activity was always found in the fractions on the descending slope of peak III. When the fractions corresponding to the particular peaks shown in Fig. 1 were suitably pooled, concentrated and subjected to electrophoresis in polyacrylamide gel at pH4.5 a characteristic pattern was always obtained (Plate 1). By slicing the non-stained gels and measuring proteolytic activity, the main proteinase was identified as two fast-migrating bands (Fig. 2). The faster-moving component is present predominantly in the fractions of the descending limb of peak III (IIIb) and this may indicate some differences in molecular weight of these components. Proteinase 1 present in peak II (Fig. 1) was probably inactivated during electrophoresis and could not be demonstrated on the basis of its enzymic activity after slicing gels.

A standardized analytical column of Sephadex G-75 was used to estimate molecular weights of these



EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis of leucocyte-granule extract fractionated on a column of Sephadex G-75

Electrophoresis was carried out in 7% gels at pH4.5. Proteins were stained with Amido Black. (*a*) Full extract of leucocyte granules; (*b*) high-molecular-weight components (peak I, Fig. 1); (*c*) proteins of peak II (Fig. 1); (*d*) proteins of the ascending slope of peak III (Fig. 1); (*e*) proteins of the descending slope of peak III (Fig. 1); (*f*) proteins of peak IV (Fig. 1).

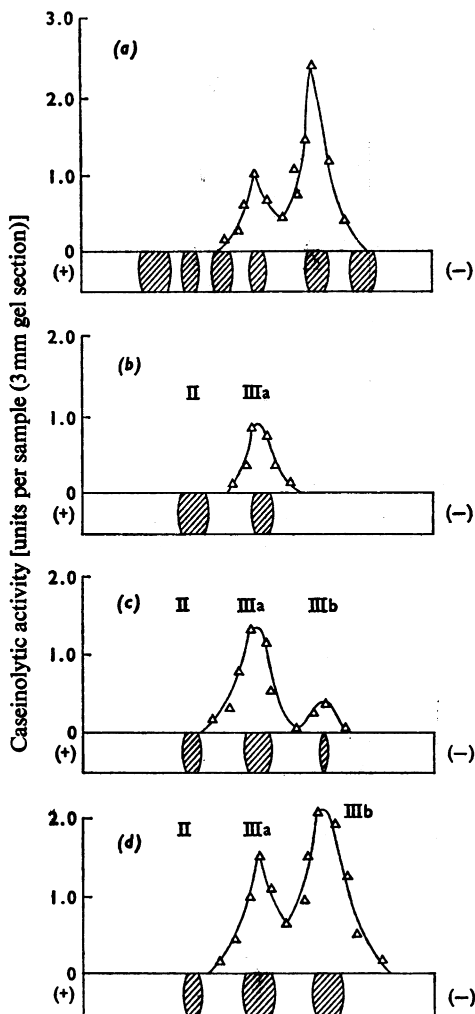


Fig. 2. *Neutral proteinase activity in the fractions of leucocyte granule extract separated by polyacrylamide-gel electrophoresis*

Non-stained gels were sliced into 3mm sections and proteinase activity was determined with casein as substrate (see the Materials and Methods section). Roman numbers refer to fractions obtained on a Sephadex G-75 column (cf. Fig. 1 and Plate 1). (*a*), Gel (*a*), Plate 1; (*b*), gel (*c*), Plate 1; (*c*), gel (*d*), Plate 1; (*d*), gel (*e*), Plate 1.

two neutral proteinases detected in the granule extract. The results indicate that the mol.wt. of proteinase 1 is approx. 38000 and of proteinase 2, about 22000. Similar values were obtained during filtration in 0.15M-NaCl or 2M-KCl. This indicates that no significant interaction of proteinase with Sephadex occurs, in contrast with lysozyme, which was significantly delayed during filtration under the conditions described above. Anomalous behaviour on Sephadex columns was reported for the rat mast-cell proteinase (Kawiak *et al.*, 1971) and for some other proteinases of bacterial origin (Voordouw *et al.*, 1974).

The molecular weight of proteinase 2 in partly purified preparation after Sephadex G-75 chromatography was also estimated by ultracentrifugation in sucrose gradients. A broad single peak of neutral proteinase activity was found in the collected fractions. Assuming a roughly spherical shape for the protein molecule and that the partial specific volume is $0.725\,cm^3/g$ (Martin & Ames, 1961), the coefficient of sedimentation was estimated as approx. 2.5×10^{-3}S, corresponding to a protein of 22000 daltons.

When the full granule extract was subjected to isoelectric focusing in the pH range 3–10, two maxima of proteolytic and esterolytic activity were found at pH5.3 and 8.8 (Fig. 3, Expt. A). By using partly purified preparations after Sephadex G-75 filtration, the first peak, pI = 5.3, was identified as proteinase 1. Preliminary data suggest that this is a distinct enzyme and not just a dimer of proteinase 2, since rechromatography of this component on Sephadex G-75 in 2M-KCl or in 5M-urea did not change the elution pattern. On the other hand, the addition of heparin to the full granule extract before isoelectric focusing shifted the peak at pH8.8 toward lower pH

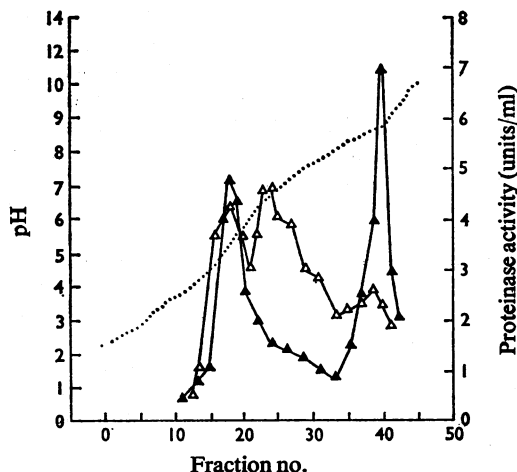


Fig. 3. *Isoelectric focusing of leucocyte-granule extract*

About 15mg of protein from the full leucocyte-granule extract was placed in a continuous sucrose gradient with 1% Ampholine. The gradient was run for 48h at 300V, 0.5mA, and 2.5ml fractions were used for the determination of pH (....) and caseinolytic activity. Expt. (A), granule extract alone; Expt. (B), after the addition of 50 units of heparin to the extract. ▲, Caseinolytic activity in Expt. (A); △, caseinolytic activity in Expt. (B).

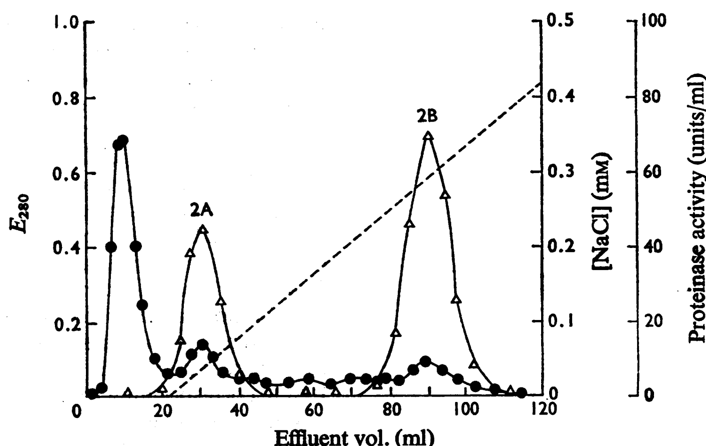


Fig. 4. *Ion-exchange chromatography of proteinase* 2

Partly purified enzyme (about 10mg of protein) after Sephadex G-75 filtration was applied to a column (2cm×20cm) of CM-Sephadex A-25 equilibrated with 0.05M-sodium phosphate buffer, pH7.4. Elution in this buffer in a linear NACl gradient (---) obtained with 50ml of 0.05M-sodium phosphate buffer, pH7.4, and 50ml of 1M-NaCl in this buffer; ●, E_{280}; △, caseinolytic activity.

values without affecting the peak at pI 5.3 (Fig. 3, Expt. B). This indicates formation of soluble complexes between heparin and proteinase 2 and confirms that proteinase 1 is a distinct protein. Although electrofocusing of the full granule extract allowed the separation of two proteinases, it could not be used on preparative scale, since the recovery of activity was always less than 20% of that applied to the column.

When the fractions containing neutral proteinase 2 eluted from Sephadex G-75 column were subjected to electrofocusing a single peak of activity at pH8.7–8.9 was always observed, whereas in polyacrylamide-gel electrophoresis two active zones were found (cf. Fig. 2). It was supposed that the second component showing a higher isoelectric point (pI presumably above 10) is inactivated during electrofocusing. This was confirmed by the experiment in which samples showing proteolytic activity after isoelectric focusing (pI8.8) were subjected to polyacrylamide-gel electrophoresis at pH4.5. Only one zone of activity corresponding to the slower-migrating component was found.

Further purification of proteinase 2 and separation of these two enzymic components differing in isoelectric point was achieved by ion-exchange chromatography (Fig. 4, Table 2). Two distinct peaks of caseinolytic activity were obtained, proteinase 2 being always more abundant. When examined by polyacrylamide-gel electrophoresis at pH4.5 the preparations 2A and 2B were homogeneous and showed relative mobilities corresponding exactly to two active zones observed on analysis of the full

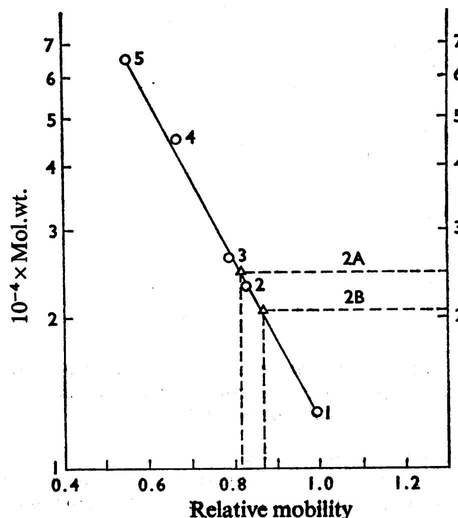


Fig. 5. *Polyacrylamide-gel electrophoresis of proteinase* 2 *in* 0.1% *sodium dodecyl sulphate*

Concentrated fractions of peaks 2A and 2B after CM-Sephadex chromatography (Fig. 4) were separated on polyacrylamide gel in 0.02M-sodium phosphate buffer, pH7.0, containing 0.1% sodium dodecyl sulphate, and then stained with Coomassie Brilliant Blue. The position of the protein zone in the gel was used to calculate a relative mobility. The following proteins served as standards: 1, cytochrome *c*; 2, chymotrypsinogen; 3, cytochrome *c* (dimer); 4, ovalbumin; 5, bovine serum albumin.

Table 2. *Purification of neutral proteinase 2 from the granule extract of horse polymorphonuclear leucocytes*

Proteinase activity was determined with casein, and esterase activity with Z-Ala-ONp as substrates (see the Materials and Methods section). Some additional 8% of proteolytic activity and 9% of esterolytic activity were recovered in the proteinase 1 peak after Sephadex G-75 chromatography.

Purification stage	Protein: Total (mg)	Protein: Yield (%)	Caseinolytic activity: Total (units)	Caseinolytic activity: Yield (%)	Caseinolytic activity: Sp. activity (units/mg)	Purification	Activity ratio $\left(\frac{\text{Esterase}}{\text{Proteinase}} \times 100\right)$
Granule extract	141.0	100.0	3807	100.0	27	1.0	1.55
Sephadex G-75	9.22	6.5	1411	37.0	153	5.6	1.38
CM-Sephadex							
Peak A	0.89	0.6	218	5.7	245	9.1	1.05
Peak B	0.68	0.5	265	6.9	390	14.4	1.89

leucocyte-granule extract (cf. Plate 1 and Fig. 2). Proteinase 2B was identified as the faster-migrating component (of higher isoelectric point) which was inactivated during isoelectric focusing.

The purity of these preparations was confirmed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Without the addition of mercaptoethanol, proteinases migrated as single bands of slightly different mobility. With suitable protein standards their mol.wts. were estimated as 24000 (proteinase 2A) and 20500 (proteinase 2B) (see Fig. 5.) The difference in molecular weight is small and probably for that reason it was not detected during gel filtration on Sephadex G-75 and during density-gradient centrifugation.

Discussion

Our experiments indicate that cytoplasmic granules of horse blood leucocytes contain at least three neutral proteinases that also show alanine nitrophenyl esterase activity. This pattern resembles human granulocytes, but mol.wts. of the enzymes in humans are in the range of 32000–36000 (Ohlsson & Olsson, 1974; Rindler-Ludwig *et al.*, 1974), whereas the values found in the present study for horse proteinases were 38000, 24000 and 20500. Isoelectric points of human leucocyte proteinases are approximately 9.4–10.8 (Janoff & Basch, 1971) whereas we found pI values of 5.3, 8.8 and above 10 for horse enzymes. Human granulocyte elastases form a strongly related protein family, since they give reaction of identity on immunodiffusion and they have similar amino acid composition (Ohlsson & Olsson, 1974). Molecular parameters of horse enzymes are more divergent, and moreover, show differences in substrate specificities [see the following paper, Koj *et al.* (1976)]. Further detailed studies are needed to establish sedimentation coefficients of horse proteinases by analytical ultracentrifugation and to determine their amino acid composition and immunological properties. It should also be pointed out that the leucocytes used in our experiments did not represent a homogeneous cell population, and it is still possible that proteinase 1 may derive from other types of leucocytes accompanying the polymorphonuclear variety.

Combined methods of subcellular fractionation, gel filtration and ion-exchange chromatography allowed us to obtain homogeneous preparations of proteinase 2. Owing to the presence of proteinase inhibitor in cell lysate, only approximate assessment of the degree of enzyme purification can be made. Taking into account the protein content of cell lysate and total recovery of enzyme in the material shown in Table 1, a specific activity of approx. 2.5–3 units/mg of protein is obtained. The final specific activity of proteinase 2A and 2B was 250–400 units/mg (Table 2) hence at least a 100-fold purification was achieved with an overall yield of about 10%.

Interaction of proteinase 2 with heparin represents an interesting phenomenon which has already been studied by Davies *et al.* (1971) for rabbit leucocyte proteinase. It appears that horse leucocyte esteroproteinases share the property of complexing with some polyanions. Such complexes are held mainly by electrostatic forces, being easily disrupted at high pH or high ionic strength. Polymorphonuclear-leucocyte granules contain an abundance of sulphated anionic polysaccharides (Olsson, 1969). Their interaction with elastase-like proteinases may contribute to the latency of these enzymes. On the other hand, polyanions may prevent inhibition of proteinases by cytoplasmic inhibitor (cf. Table 1).

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