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PURIFICATION AND SOME PROPERTIES OF A NEUTRAL PROTEASE FROM HUMAN LEUKOCYTE GRANULES AND ITS COMPARISON WITH PANCREATIC ELASTASE

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Summary

1. A cationic protease has been purified from the granule fraction of blood-donor leukocytes by a preparative method including precipitation by acetone and chromatography on Bio-Gel A 1.5 m, CM-Sephadex C-50 and Sephadex G-G-75.

2. The pH optimum against denatured bovine hemoglobin is 7.4. Gel chromatography indicated a molecular weight close to 23 000.

3. This neutral protease (EC 3.4.-.-) is able to split the synthetic esters Z-Ala-NPh and $AcAla_3OMe$, its activity on the former substrate being 2.2 times greater than that of pancreatic elastase, on the latter the same. It differs crucially from pancreatic elastase in having small elastinolytic activity.

4. In cationic disk electrophoresis, neutral protease resolves into three protein bands with lower mobility than lysozyme: all bands exhibit esterolytic activity against 2-acetoxy-3-naphthoic acid *o*-toluidide, strongly suggesting that they represent isoenzymes.

5. The enzyme is completely inhibited by iPr_2P-F, partially so by soybean trypsin inhibitor and Trasylol. Cysteine, EDTA and $TosLysCH_2Cl$ have no effect.

6. During chromatograpy on CM-Sephadex C-50 a more positively charged enzyme(s) was identified. This had hemoglobinolytic activity at pH 7.4 but only a small esterolytic effect on Z-Ala-NPh; it showed only traces of activity against $AcAla_3OMe$.

All acute inflammatory processes are accompanied by the infiltration of polymorphonuclear leukocytes. One of the important and beneficial functions of

The following abbreviations are used in this communication: Hb = bovine hemoglobin; Z-Ala-NPh = *N*-t-BOC-L-alanine *p*-nitrophenyl ester; $AcAla_3OMe$ = *N*-acetyl-L-alanyl-L-alanyl-L-alanine methyl ester; $TosLysCH_2Cl$ = *N*-α-*p*-tosyl-L-lysine chloromethyl ketone · HCl; iPr_2P-F = di-*iso*propyl fluorophosphate.

polymorphonuclear leukocytes is the phagocytosis and breakdown of tissue-damaging substances. In addition to this role it is now known that under certain conditions polymorphonuclear leukocytes can damage host tissue. One possible mechanism of this might be the extracellular release of the lysosomal enzymes during phagocytosis in large enough quantities [1—3]. During the last few years it has been found that viable polymorphonuclear leukocytes secrete these enzymes, even during so-called nonphagocytic enzyme release [4], and also a protease which splits off proteoglycan from cartilage at pH 7.4 [5].

Over the last two years a number of papers have been published describing proteases from human polymorphonuclear leukocytes active at neutral pH. Using the affinity chromatography method, Janoff [6] obtained purified elastase from human granulocytes. An enzyme with a very high specific elastolytic activity was obtained from the same source by Ohlsson and Olsson [7]. In human polymorphonuclear leukocytes, Schmidt and Havemann [8] and Rindler-Ludwig et al. [9,10] identified and partially purified enzymes showing broad proteolytic and esterolytic activity at neutral pH. The subcellular localization of such activities in human polymorphonuclear leukocytes was demonstrated by Dewald et al. [11], who found that proteolytic activity connected with esterolytic activity was located exclusively in the azurophil granules.

This paper describes a neutral protease obtained from human leukocytes. Some of its properties have been investigated and compared with those of known proteolytic enzymes.

Material and Methods

Substances. Bovine hemoglobin type II, *N*-t-BOC-L-alanine *p*-nitrophenyl ester, *N*-acetyl-L-alanyl-L-alanyl-L-alanine methyl ester, 2-acetoxy-3-naphthoic acid *o*-toluidide Sigma N 2875, elastin-orcein, elastase type III from hog pancreas, lysozyme from egg white grade I, α-chymotrypsin from bovine pancreas type II. Fast Blue RR salt, trypsin inhibitor from soybean type II-S, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone · HCl (Sigma Chemical Company, St. Louis, Mo., USA); elastin powder, di-*iso*propyl fluorophosphate (Fluka AG, Buchs, Switzerland); Trasylol (Bayer, Leverkusen, GFR). All other substances were of analytical grade.

Preparation and extraction of leukocyte granules; precipitation by acetone. For isolation of leukocytes the buffy coats of 18 l of fresh donor blood were subjected to dextran sedimentation and hypotonic shock at 2° C [12]. The platelet-free leukocytes were suspended in 0.2 M Tris-HCl pH 7.4 and homogenized. The granular pellet obtained by differential centrifugation between 400 × *g* for 10 min and 25 000 × *g* for 30 min was suspended in 55 ml 0.8 M KCl + 0.2 M Tris · HCl (pH 7.4) and subjected to 10 cycles of freeze-thawing. The clear supernatant was brought to precipitation by acetone up to a final concentration of 30%. Protein was determined by absorbance at 280 nm or by the method of Lowry et al. at 750 nm [13].

Enzyme assays

Proteolytic activity. 1 ml of an 8% hemoglobin solution prepared in 5 M urea

+ 0.2 Tris · HCl (pH 7.4) was mixed with 1 ml 5 M urea + 0.8 M KCl + 0.2 M Tris · HCl (pH 7.4). After addition of 0.2 ml of the test sample, the mixture was incubated on an agitation water bath at 37° C for 3 h. The reaction was terminated by addition of 4 ml 5% trichloroacetic acid. For controls, tubes containing substrate with buffer or enzyme only were incubated separately and mixed together just before the trichloroacetic acid was added. One unit of hemoglobinolytic activity was defined as that quantity of enzyme which caused an increase in the absorbance at 280 nm of 1.0 per h.

Esterase activity. (a) Esterolytic activity against Z-Ala-NPh was measured according to the method of Visser and Blout [14]. Briefly, 50 µl acetonitrile containing the substrate (final concentration 0.19 mM) was added to the reaction mixture containing 3 ml of 0.05 M phosphate buffer pH 6.5 and the test sample (10—100 µl containing 2—100 µg protein). The increase in absorbance at 347.5 nm occurring during the first 5 min as compared with a control prepared without enzyme was measured at 25° C. One unit of esterase activity was defined as that quantity of enzyme causing an increase of the absorbance amounting to 1.0 per min. Inhibition tests were carried out by adding the enzyme to a solution of inhibitor in the same buffer. After pre-incubation at 25° C for 10 min the substrate was added. Controls were prepared similarly but without enzyme.

(b) Esterolytic activity against $AcAla_3OMe$ as substrate was measured using essentially the method of Gertler and Hofmann [15] but with a higher concentration of salts. The substrate (final concentration 5.2 mM) was dissolved in 2 ml 2 mM Tris buffer adjusted to pH 8.0 with HCl and containing 1.0 M NaCl. To this mixture a sample (10—100 µl) was added. Titration was carried out with 0.002 M M NaOH at 25° C using a pH-stat equipped qith an automatic titrator (Radiometer Inc., Copenhagen, Denmark). Before assay, all samples were dialyzed, commercial enzymes being dissolved in the above-mentioned buffer. One unit of activity was defined as the liberation of nmol of H^+ per minute.

Elastinolytic activity. This was measured essentially by the method of Sachar et al. [16] using 30 mg elastin or 20 mg elastin-orcein suspended in 0.1 M Tris · HCl pH 8.8. The test samples, i.e., granular extract, purified neutral protease and commercial enzymes, were dialyzed or freshly solubilized in the same buffer. After incubation of the mixtures (final volume 3 ml) at 37° C for 1 h the reaction was stopped by addition of 2 ml cold 0.7 M phosphate buffer (pH 6.0). The enzyme activity was measured as the absorbance of the clear supernatant at 276 nm (elastin) or 590 nm (elastin-orcein). Blanks were prepared with enzyme and substrate incubated separately and combined after the addition of 0.7 M phosphate buffer. For control of neutral protease inactivation during incubation, equal amounts of dialysed neutral protease were incubated in 0.1 M Tris · HCl ph 8.8 but without substrate, and the activity against Z-Ala-NPh measured.

Cationic disk electrophoresis. This was carried out by the method of Reisfeld et al. [17] at pH 5.0 and 5.9 in the small- and large-pore gels respectively. Triton-X-100 at a concentration of 0.5% was added to the system when necessary. Staining for protein was carried out with Coomassie Brilliant Blue R, for esterolytic activity with 2-acetoxy-3-naphthoic acid *o*-toluidide [11].

Results

Purification of the neutral protease. The purification data for the clear supernatant of granule extract are summarized in Table I. Each step in the purification was followed by hemoglobin and Z-Ala-NPh assays. The conditions and results for the three chromatographic steps of purification as outlined in Table I are presented in Figs. 1—3.

Disk electrophoresis. Cationic disk electrophoresis without Triton X-100 resulted in the separation of four protein bands, three major and one minor, with electrophoretic mobilities lower than that of lysozyme (Fig. 4). Three of these bands (gels a and d: bands 1, 2, 4) showed distinct esterolytic activity with 2-acetoxy-3-naphthoic acid *o*-toluidide, but the minor protein band 3 could not be attributed to a distinct esterase band owing to diffusion of the stain. Disk electrophoresis in the presence of Triton X-100 resulted in three protein bands, all of which exhibited esterolytic activity.

Criteria of purity. After sieve filtration on Sephadex G-75 only one peak of protein was eluted. All resulting fractions exhibited the same specific hemoglobinolytic and esterolytic activity (Fig. 3). In addition, cationic disk electrophoresis in the presence of Triton X-100 (Fig. 4) resolved the enzyme onto three protein bands each giving a positive reaction with the protease substrate 2-acetoxy-3-naphthoic acid *o*-toluidide. The protein and protease bands exhibited the same relative intensity. From these results it can be concluded that the enzyme

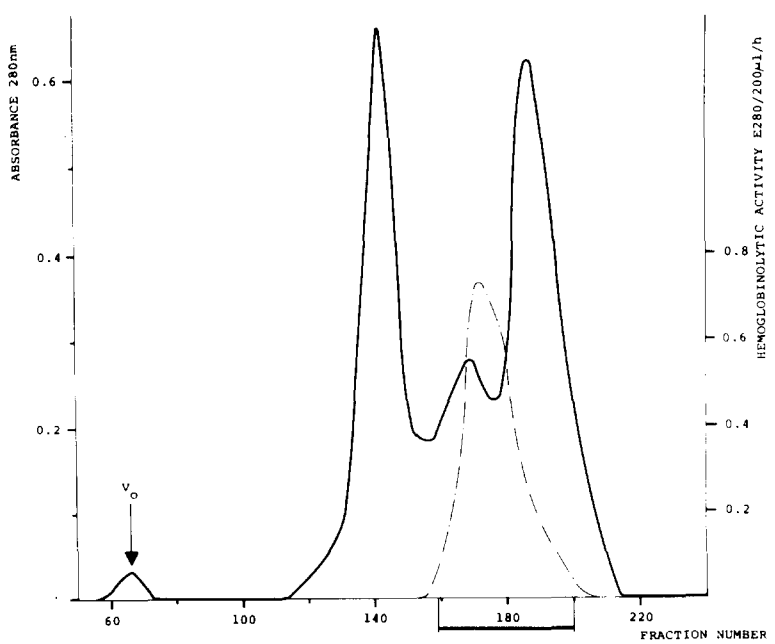

Fig. 1. Sieve filtration on Bio-Gel A 1.5 m. Column 5 × 80 cm, equilibrated with 0.2 M Tris · HCl buffer, pH 7.4 containing 0.8 M KCl. Flow rate 60 ml/h; 9.0 ml fractions. 15 ml of dialysed against the same buffer and concentrated supernatant (250 mg protein, measured as absorbance at 280 nm) was applied after precipitation with acetone. - · - · -, Hemoglobinolytic activity; ———, Absorbance at 280 nm; V_O void volume.

TABLE I

PURIFICATION OF NEUTRAL PROTEASE FROM HUMAN LEUKOCYTE GRANULES

	Hemoglobin (Hb)					Z-Ala-NPh			
	Protein * (mg)	Total activity (units)	Specific activity	Yield (%)	Purification factor	Total activity (units)	Specific activity units/mg	Yield (%)	Purification factor
Granule extract (corresponding to approx. $4 \cdot 10^{10}$ cells)**	470	770	1.64	100	1	109	0.232	100	1
Acetone precipitation ***	250	731	2.92	95	1.8	151	0.604	138	2.6
Bio-Gel A 1.5 m	113	624	5.52	81	3.4	130	1.15	119	5
CM-Sephadex C-50 peak I (linear gradient elution)	8.3	260	31	34	19	63.4	7.6	58	33
Sephadex G-75	5.9	189	32	25	20	49.8	8.4	46	36

* Calculated from volume and E_{280}.

** Counted after dextran sedimentation; polymorphonuclear leucytes content 65%.

*** 22 ml of acetone cooled to −70°C was added to 52 ml of granule extract.

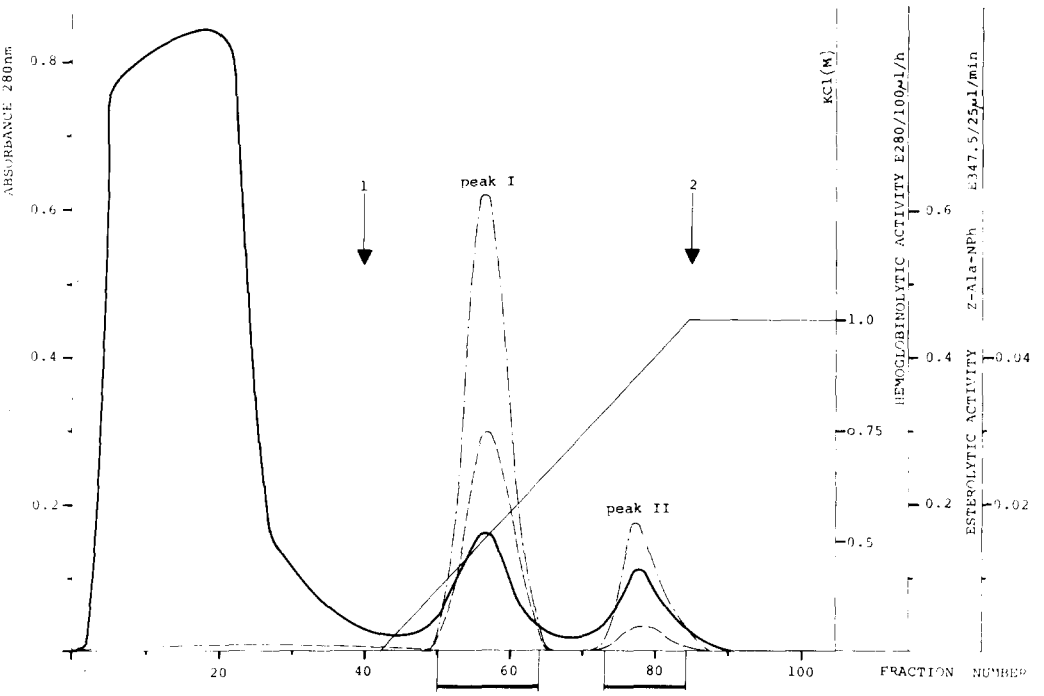

Fig. 2. Chromatography on CM-Sephadex C-50. Column 2.5 × 8 cm, equilibrated with 20 mM Tris · HCl buffer, pH 8.5, containing 250 mM KCl. Flow rate 120 ml/h; 6 ml fractions. 120 ml of pooled and concentrated material from Bio-Gel A 1.5 m (113 mg protein measured as absorbance at 280 nm) was applied after dialysis against equilibrating buffer. Arrow 1 indicates start of a linear gradient composed of 130 ml of equilibrating buffer and 130 ml of 20 mM Tris · HCl pH 8.5 containing 1 M KCl. Arrow 2 indicates change to the last buffer. - - - - - -, Esterolytic activity (Z-Ala-Nph). · · · · · ·, Hemoglobinolytic activity; ———, Absorbance at 280 nm.

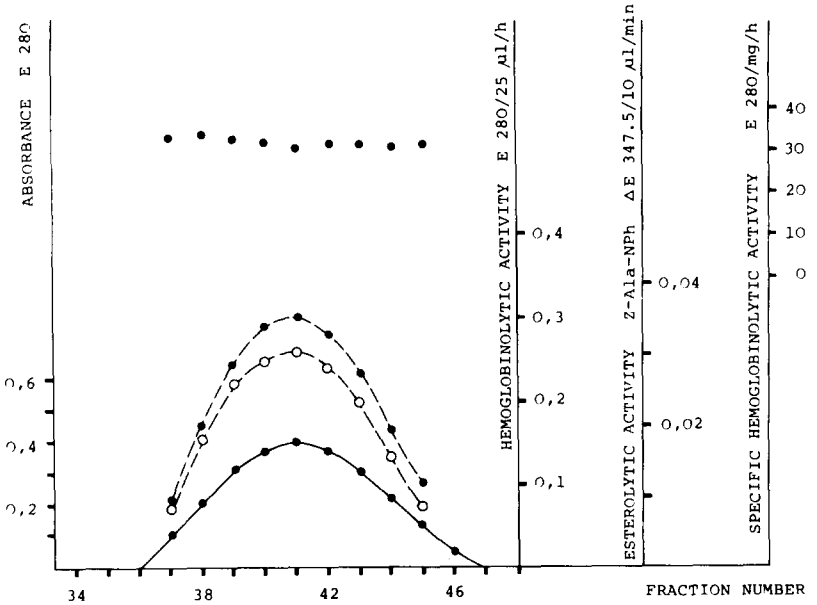

Fig. 3. Sieve filtration on Sephadex G-75. Column 1.5 × 90 cm equilibrated with 20 mM Tris · HCl buffer pH 7.4 containing 0.5 M KCl. Flow rate 8 ml/h; 2.4 ml fraction. 3 ml of concentrated peak I fractions (see Fig. 2), equal to 8.3 protein, was applied. -○-○-, Esterolytic activity (Z-Ala-NPh); · · · · · ·, Hemoglobinolytic activity; ———, Absorbance at 280 nm. Points at the top of the figure give the specific hemoglobinolytic activity in the fractions.

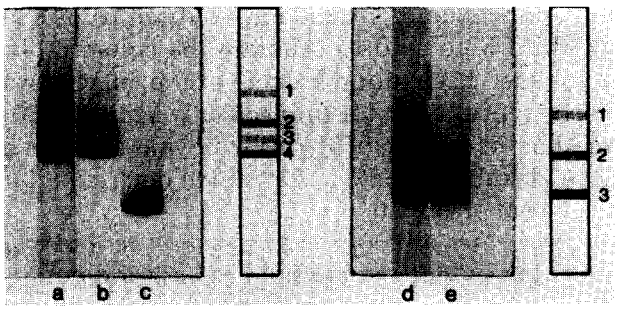

Fig. 4. Cationic disc electrophoresis of purified enzyme pooled from the Sephadex G-75 column. 10μg protein in 15% polyacrylamide gels with (gels d,e) and without (gels a-c) 0.5% Triton X-100. Gels a and d: staining for esterolytic activity with 2-acetoxy-3-naphthoic acid *o*-toluidide. Gels b and e: staining for protein with Coomassie Blue. Gel c: staining for protein of 8 μg lysozyme run for comparison. Migration form the anode (above) to the cathode. Schemes refer to protein bands in gels b and e.

was purified, that it possesses both proteolytic and esterolytic activity and that it consists of three isoenzymes.

Effect of pH on enzyme activity. The pH value for optimum activity of the purified enzyme with hemoglobin as substrate was found to be 7.4 (Fig. 5). In experiments with Z-Ala-NPh as substrate, a similar curve was obtained. Higher concentrations of phosphate buffer or KCl up to 0.5 M had no effect on enzyme activity.

Molecular weight and specific extinction coefficient. Using gel filtration on Sephadex G-100, a molecular weight of approximately 23 000 was found for the

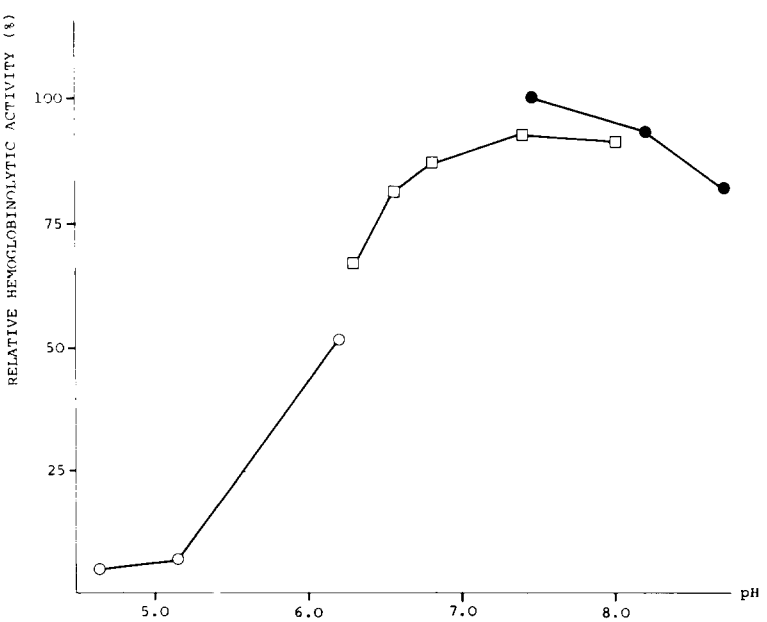

Fig. 5. Effect of pH on hemoglobinolytic activity of purified neutral protease. 20 μg of purified enzyme was used. Incubation time 3 h. Buffers were acetate (o-o-o), phosphate (□-□-□), Tris-HCl (●-●-●), each at 0.1 M concentration containing 0.2 M KCl and 5.0 M urea.

purified neutral protease. Bovine serum albumin, ovalbumin, soybean trypsin inhibitor and lysozyme from egg-white were used to calibrate the column, blue dextran to determine the void volume. The elution volume of the enzyme was slightly lower than that of soybean trypsin inhibitor (molecular weight 21 500). When the value given by the method of Lowry [13] (using bovine albumin as standard) was compared with the absorbance $E^{1\,cm}_{280\,nm}$ for proten determination the calculated coefficient for the enzyme was 1.0.

Activity of the neutral protease and other proteolytic enzymes on various substrates. In order to determine to what extent the neutral protease and porcine elastase were similar the two enzymes were tested on a variety of substrates used for determination of pancreatic elastase (Table I). In these experiments α-chymotrypsin was used a known non-elastinolytic enzyme. The values for porcine elastase have been assigned the value 100. On the basis, the purified neutral protease showed small activities of 3.6 and 8.1 on two elastin substrates. When corrected for enzyme inactivation (with Z-Ala-NPh as substrate) during dialysis (15% loss) and possibly incubation (at the end of incubation, enzyme activity in the control without substrate had fallen a further 30%), these values would increase to maximally 6.5 and 14.7 respectively. The two enzymes had the same activity with respect to $AcAla_3OMe$. With Z-Ala-NPh the activity of neutral protease was about 2.2 times greater. α-Chymotrypsin likewise showed some marked difference compared with neutral protease. On two synthetic substrates (Z-Ala-NPh and $AcAl_3OMe$) its activity was respectively 10 and 16 times smaller than that of the neutral protease. Comparison of the crude granular extract with the purified neutral protease showed almost identical purification factors for activity against elastin and Z-Ala-NPh.

Effect of inhibitors. A linear relationship was found between the concentration of the neutral protease (up to 8 μg protein per assay) and the color yield when Z-Ala-NPh was used as substrate. Using the same substrate, the enzyme

TABLE II

RELATIVE ACTIVITY OF NEUTRAL PROTEASE AND OTHER PROTEOLYTIC ENZYMES AGAINST VARIOUS SUBSTRATES

Values for porcine elastase have been arbitrarily set at 100; values for other enzymes relate to the same amount of protein as for porcine elastase. Numbers in brackets are amounts of protein used for assay. Assay procedures are as described under Material and Methods.

Substrate	Granular extract	Neutral protease	Porcine elastase	α-Chymotrypsin
Elastin-orcein	0.00 *	3.6 *	100	0.62
	(1120 μg)	(120 mg)	(40 μg)	(200 μg)
Elastin	0.24 *	8.1 *	100	1.8
	(1120 μg)	(120 μg)	(40 μg)	(400 μg)
Z-Ala-NPh	6.1	221	100	23.2
	(56 μg)	(2.4 μg)	(4 μg)	(16 μg)
$AcAla_3OMe$	2.6	95.7	100	6.0
	(168 μg)	(4 μg)	(4 μg)	(80 μg)

* Losses of enzyme activity occuring during dialysis and incubation are not considered, see text.

TABLE III

EFFECT OF POTENTIAL INHIBITORS ON NEUTRAL PROTEASE

3.6 μg of the enzyme was used in the assay against Z-Ala-Nph as substrate.

Compound	Final concn. (mM)	Inhibition (%)
iPr_2P-F	0.01	100
Soybean trypsin inhibitor	1.0 mg/ml	100
	0.1 mg/ml	96
Trasylol	1.0	68
$TosLysCH_2Cl$	1.0	0
EDTA	1.0	0
Cysteine · HCl	0.1	0

was tested in the presence of typical inhibitors of proteolytic enzymes, namely iPr_2P-F, soybean trypsin inhibitor, Trasylol, $TosLysCH_2Cl$ and EDTA, as well as cysteine under the conditions given in Table III. The most effective inhibitor was iPr_2P-F, which completely abolished neutral protease activity at a concentration of 10^{-5} M. Soybean trypsin inhibitor (0.1 mg/ml) and Trasylol (10^{-3} M), also had a distinct inhibitor effect. $TosLysCH_2Cl$, EDTA and cysteine had no effect on the enzyme.

Discussion

The entire purification procedure for neutral protease yielded 25% of the hemoglobinolytic activity of the crude extract. It follows that only about 5% of the extracted granule protein consists of neutral protease.

Neither cysteine nor EDTA had any effect on the activity of the enzyme, so that it is neither a thiol- nor a metalloproteinase. Its inhibition by iPr_2-P-F (Table III) would put neutral protease among the serine proteases. Neutral protease carries a positive charge and has a somewhat lower electrophoretic mobility than lysozyme (Fig. 4), a property distinguishing it from the chymotrypsin-like proteases described by Rindler-Ludwig and Braunsteiner [10] and by Schmidt and Havemann [8].

Neutral protease has similarities to pancreatic elastase in respect of its cationic nature and esterolytic properties against the elastase substrates $AcAla_3OMe$ and Z-Ala-NPh. Its activity on the latter substrate was 2.2 times greater than that of pancreatic elastase, on the former the same.

Using dyed or undyed elastin under the assay conditions described by Sacher et al. [17], neutral protease was found to possess only 3.6 and 8.1% respectively of specific pancreatic elastase activity (Table II). The corresponding activities of neutral protease corrected for losses during dialysis and possibly incubation would maximally amount to 6.5 or 14.7% respectively. In fact these activities might even be smaller for the following reasons: first, inactivation of neutral protease during incubation in the presence of elastin is probably less than that measured in the control tube containing no substrate and used for this calculation; secondly, a possible inactivation of pancreatic elastase during incubation was not considered; thirdly, in view of the fact that α-chymotrypsin

also gave low elastinolytic values (Table II), the result obtained with neutral protease may be partly due to substrate impurity. In conclusion, under conditions favorable to elastase the neutral protease purified by us exhibits elastinolytic activities too small to justify its classification as an elastase, although it has important properties in common with pancreatic elastase. A similar conclusion was arrived at by Mallory and Travis [20] in respect of an anionic pancreatic protease with activity against typical synthetic elastase substrates but no elastinolytic activity.

Neutral protease resembles the granulocyte elastinolytic enzymes described by Janoff [6], Ohlsson and Olsson [7] and Schmidt and Havemann [8] in respect of its affinity for particular esters, its cationic nature [6-8] and its sensitivity to iPr_2P-F [18] and soybean trypsin inhibitor [19]. However, neutral protease differs from Janoff's enzyme [6] mainly in having a four-fold smaller activity on dyed elastin. On the assumption therefore of strictly identical conditions for determination of elastinolytic activity, these enzymes appear to be different.

In the light of its properties, our neutral protease would seem to be very similar to the protease partially purified from myeloid leukocytes by Rindler-Ludwig et al. [9,21], although it must be borne in mind that their enzyme was obtained from leukemic cells. Our neutral protease has been found to have a molecular weight of 23 000, which is 10 000 less than that of the Rindler-Ludwig enzyme [9].

In addition to neutral protease, the granule extract contained a further proteolytic enzymes (or enzymes). This appeared in peak II after CM-Sephadex C-50 separation (Fig. 2) and differed clearly from neutral protease in having a higher cationic charge, a lower specific activity against Hb and Z-Ala-NPh and only traces of activity against $AcAla_3OMe$ (Table IV). These properties suggest that peak II comprises the chymotrypsin-like enzyme or enzymes isolated by Rindler-Ludwig and Braunsteiner [10] and by Schmidt and Havemann [8].

Preliminary experiments have indicated that neutral protease attacks human immunoglobulin G and cartilage proteoglycan in vitro. It is therefore suggested that the neutral protease described here may have important biological functions.

TABLE IV

PROTEOLYTIC AND ESTEROLYTIC ACTIVITY OF POOLED PEAKS I AND II OBTAINED BY CHROMATOGRAPHY ON CM-SEPHADEX C-50

Substrate	Peak I (8.3 mg protein) *		Peak II (4.4 mg protein) *	
	Total activity (units)	Specific activity (units/mg)	Total activity (units)	Specific activity (units/mg)
Hb	260	31	76	17
Z-Ala-NPh	63.4	7.6	6.2	1.4
$AcAla_3OMe$	666	80	6.4	1.4

* Claculated from volume and E_{280}.

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References

1 Weissmann, G., Zurier, R.B., Spieler, P.J. and Goldstein, I.M. (1971) J. Exp. Med. 134, 149s—165s
2 Baehner, R., Karnovsky, M.J. and Karnovsky, M.L. (1968) J. Clin. Invest. 47, 187—192
3 Weissmann, G. and Rita, G.A. (1972) Nat. New Biol. 240, 167—172
4 Henson, P.M. (1971) J. Exp. Med. 134, 114s—135s
5 Oronsky, A., Ignarro, L. and Perper, R. (1973) J. Exp. Med. 138, 461—472
6 Janoff, A. (1973) Lab. Invest. 29, 458—464
7 Ohlsson, K. and Olsson, I. (1974) Eur. J. Biochem. 42, 519—527
8 Schmidt, W. and Havemann, K. (1974) Hoppe-Seyler's Z. Physiol. Chem. 355, 1077—1082
9 Rindler-Ludwig, R., Schmalzl, F. and Braunsteiner, H. (1974) Brit. J. Haematol. 27, 57—64
10 Rindler-Ludwig, R. and Braunsteiner, H. (1975) Biochim. Biophys. Acta 379, 606—617
11 Dewald, B., Rindler-Ludwig, R. Bretz, U. and Baggiolini, M. (1975) J. Exp. Med. 141, 709—723
12 Fallon, H.J., Frei, E., Davidson, J.D., Trier, J.S. and Burk, D. (1962) J. Lab. Clin. Med. 59, 779—791
13 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1959) J. Biol. Chem. 193, 265—275
14 Visser, L. and Blout, E.R. (1972) Biochim. Biophys. Acta 268, 257—260
15 Gertler, A. and Hofmann, T. (1970) Can. J. Biochem. 48, 384—386
16 Sachar, L.A., Winter, K.K., Sicher, N. and Frankel, S. (1955) Proc. Soc. Exp. Biol. Med. 90, 323—326
17 Reisfeld, R.A., Lewis, U.J. and Williams, D.E. (1962) 195, 281—283
18 Janoff, A. and Zeligs, J.D. (1968) Science 161, 702—704
19 Janoff, A. and Scherer, J. (1968) J. Exp. Med. 128, 1137—1151
20 Mallory, P.A. and Travis, J. (1975) Biochem. 14, 722—730
21 Rindler, R. and Braunsteiner, H. (1973) Blut 27, 26—32