Biochimica et Biophysica Acta, 480 (1977) 228—240

BBA 67999

ISOLATION OF CATHEPSIN D FROM HUMAN LEUCOCYTES

ISAO ISHIKAWA and GIORGIO CIMASONI

School of Dentistry, University of Geneva 19, rue Barthelemy-Menn, 1211 Geneva (Switzerland)

(Received January 28th, 1976)
(Revised manuscript received August 23rd, 1976)

Summary

Acid and neutral protease activities were determined in the granule fractions of polymorpho and mononuclear leucocytes, separated from human blood by means of a discontinuous density gradient centrifugation. The mononuclear leucocytes contained only acid protease while preparations from polymorphonuclear leucocytes showed a predominant activity at neutral pH with a small peak in the acid range. A separation of the acid from the neutral enzyme could be obtained in the granule fraction of polymorphonuclear leucocytes by means of DEAE chromatography. The acid enzyme was then purified from a mixture of leucocytes, more than 400 times, by means of gel chromatography with Sephadex G-200 superfine. The purified acid protease showed an optimum pH of 3.6, had a molecular weight at 42 000 and was characterized by a single protein band (R_f = 0.31) by disc-gel electrophoresis. With all probability this enzyme can be classified as cathepsin D (EC 3.4.4.23).

Introduction

The liberation of proteases from the various cells accumulating during the process of inflammation could play a significant role in tissue destruction [1]. For instance, cathepsin D (EC 3.4.4.23) and cathepsin BI (EC 3.4.4.-) have been shown to be largely responsible for the degradation of articular cartilage [2,3]. In our laboratory, cathepsin D has been identified in the exudate from inflamed gingivae, in concentrations correlated to the severity of the tissue destruction [4]. Recently, working with homogenates of tissue, we found that inflamed gingivae contain more of the free portion of lysosomal enzymes, including cathepsin D [5].

The relative contribution of various types of cells to the liberation of proteases in an inflamed area is still unknown. With regard to polymorphonuclear leucocytes, extensive investigations have shown that human polymorphonuclear

leucocytes contain proteolytic enzymes with optimum activity at neutral pH, capable of degrading a wide variety of tissue components [6—9]. However, the presence of acid proteases in human polymorphonuclear leucocytes has been almost ignored and, recently, even denied [10]. Two reasons could explain this situation. Firstly, the purification of polymorphonuclear leucocytes being an intricate task, contaminations by mononuclear leucocytes can supply significant amounts of acid enzyme to the polymorphonuclear leucocyte preparations. Second, when trying to identify the neutral and the acid enzyme by means of pH dependence, no clear peak is obtained in the acid range, but there is an almost continuous increase of activity towards neutrality, because of the predominance of the neutral protease.

In the present experiment the activities of acid and neutral proteases have first been measured in subcellular fractions of polymorpho and mononuclear leucocytes from human blood. In granule fractions of highly purified human polymorphonuclear leucocytes, the acid and the neutral enzymes could clearly be separated by ion exchange chromatography. Cathepsin D was then further purified and characterized using preparations of whole leucocytes.

Materials and Methods

Isolation of polymorpho and mononuclear leucocytes from human blood

Polymorphonuclear leucocytes were purified from normal donor blood, according to the standard technique of Boyum [11]. Each time, about 200 ml of blood were used; they were prevented from coagulating by the addition of heparin (10 I.U./ml) and divided into aliquots of 30 ml. Each aliquot was then layered over 15 ml of an aqueus solution containing Isopaque and Ficoll in a 50 ml tube. The mixture of Isopaque and Ficoll was prepared by mixing 10 parts of Isopaque, 33.9% (Nyegaard and Co, Oslo, Norway) with 24 parts Ficoll 9%. The tubes were centrifuged for 20 min at 700 $\times$ *g* at room temperature. After centrifugation, the following layers could be found from top to bottom (Boyum, ref. 11): plasma, mononuclear leucocytes, a mixture of Ficoll and Isopaque and a mixture of erythrocytes and polymorphonuclear leucocytes. The layers of plasma and that containing the polymorphonuclear leucocytes were then pooled; this mixture was added to a 5% dextran solution in 0.9% NaCl, in the proportion 5 : 1. The erythrocytes were allowed to settle for one hour and the polymorphonuclear leucocytes obtained by centrifuging the dextran supernate for 8 min at 120 $\times$ *g*. The few erythrocytes which still remained were completely removed by additional hypotonic lysis [18]. The polymorphonuclear leucocytes were washed three times in 0.9% NaCl solution and once on 0.34 M sucrose. A sample of the NaCl washed leucocytes, prior to washing in the sucrose, was taken for staining with the May-Grunwald-Gimsa stain and for counting. In some later experiments, when the characterization of cathepsin D from polymorphonuclear leucocytes was already performed, the Ficoll-Isopaque solution was no longer used, and the polymorphonuclear leucocytes separated only with dextran.

Differential centrifugation

The pellet obtained after the final sucrose washing was resuspended in 3 ml

of 0.34 M sucrose solution and homogenized in a Potter-Elvehjem homogenizer in an ice-cold bath 5 times for one min each time, with a 45-s pause between each burst. After homogenization three subcellular fractions were obtained by differential centrifugation: the nuclear fraction (700 × *g* for 10 min), the granule fraction (20 000 × *g* for 20 min at 4° C) and the post-granule supernate. The granule fraction was resuspended in 2 ml of either distilled water or 50 mM Tris · HCl buffer (pH 7.8) containing 0.1% Triton X-100. The water-suspended granules were used immediately for the enzyme assays, while the granules suspended in the buffer could be stored at −20° C until use. The pellet of the nuclear fraction was stored in 2 ml of Tris · HCl buffer and the post-granule supernate also stored in the presence of 0.1% Triton X-100. The Tris buffer had been calibrated at room temperature (pH 7.8) and at 4° C (pH 7.7).

Extraction of enzymes from polymorphonuclear leucocytes granules

The stored granules obtained from 1000 ml of blood were thawed and the proteins precipitated by the addition of cold acetone (75%, v/v). The precipitate was collected by centrifugation (5000 × *g* for 15 min at 4° C) and resuspended in 10 ml of the same Tris · HCl buffer, containing 10 mM EDTA 2 Na. The suspension was dialyzed against EDTA Tris · HCl buffer for 24 h, and then against Tris · HCl buffer for another 24 h [13]. The suspension was centrifuged at 5000 × *g* for 15 min at 4° C. The clear yellowish supernate was used as a crude enzyme solution.

In the early part of our work, freezing and thawing had been used, instead of Triton X-100, in attempts to liberate the enzyme. In this case, the granules suspended in Tris · HCl buffer were freezed and thawed ten times. They were then centrifuged at 20 000 × *g* for 20 min and the supernate used as a crude enzyme solution.

Biochemical assays

Acid and neutral proteases were determined at increasing pH values using a modification of the technique of Anson [13] in the following 0.1 M buffer solutions: glycine/HCl (pH range 2.5 to 3.2), citric acid/sodium citrate (pH 3.3 to 5.5), phosphate (pH 6 to 7.8) and sodium carbonate/sodium bicarbonate (pH 9 to 10).

0.5 ml of citric acid/sodium citrate buffer (pH 3.3) was mixed with 0.2 ml of a 6% haemoglobin (Sigma type II, final concentration 1.5%) solution and with 0.1 ml of the enzyme solution (final pH 3.6); the mixture was then incubated for 3 h at 38° C. In order to avoid precipitation of haemoglobin at pH values higher than 5, the haemoglobin was dissolved in distilled water and the solution dialyzed against water. After the 3-h incubation, 3 ml of 0.3 N cold trichloroacetic acid were added to the mixture which were then filtered. Blank assays were run in the same conditions, with 0.1 ml of the enzyme solution added after the trichloroacetic acid. The differences of absorbance at 280 nm (ΔA_{280nm}) between experiment and blank filtrates were used as a measure of protease activity. In some cases, the analysis in the filtrate (0.5 ml) was done by using the Folin-Ciocalteau method at 750 nm.

The relationship between enzyme concentration and ΔE_{280nm} was linear up to 0.3 unit, and all our assays were performed within these conditions.

The specific activity of the enzyme was expressed as a difference in absorbance at either 280 nm or 750 nm per mg of protein.

Inhibition of the enzyme by serum was studied by adding serum at the concentration of 20% (w/v) in the buffer.

β-Glucuronidase (EC 3.2.1.31) was determined by the fluorometric method of Mead et al. [15] using methyl-umbelliferyl-glucuronide as substrate.

Protein concentrations were determined by the method of Lowry et al. [16]. In the process of chromatography, proteins were also analyzed by measuring the absorbance at 260 and 280 nm [17].

Concentration

For the gel chromatography, the enzyme solutions were concentrated to a volume of about 2 ml by using a dialysis membrane (Union Carbide Co.). The dialysis bag, firmly attached to a funnel, was inserted into a filter flask and submitted to vacuum overnight. For the disc gel electrophoresis, enzyme solutions were concentrated by using Amicon A-25 to a volume of about 75 μl.

Polyacrylamide gel electrophoresis

The disc electrophoresis (pH 8.9), in gels containing 7% acrylamide, was prepared according to the method of Davis [18]. Electrophoresis was carried out at 2 mamp per tube at 4°C until the tracking dye reached the bottom of the tube.

Protein staining was done with an 0.05% solution of Coomasie brilliant blue R 250 in 12.5% trichloroacetic acid according to the method of Chrambach et al. [19].

Stained gels were traced by densitometer and the R_f value was calculated. The gels were cut at intervals of 1.6 mm, the slices incubated in 0.5 ml distilled water at 4°C overnight and 0.2 ml of each solution was taken for enzyme assay.

Estimation of molecular weight

In order to obtain an estimate of the molecular weight of our purified cathepsin D preparation, a column of Sephadex G-200 superfine was used (1.6 cm × 70 cm). It was first calibrated with solutions of blue dextran 2000, aldolase (molecular weight 158 000), bovine serum albumin (67 000), ovalbumin (45 000), chymotrypsinogen A (25 000) and myoglobin (18 000). Its void volume was determined by means of blue dextran and the K_{av} calculated [21].

Results

Isolation of polymorpho and mononuclear leucocytes

Table I shows the differential counts for polymorpho and mononuclear leucocyte preparations, obtained by Ficoll-Isopaque density gradient centrifugation or single dextran sedimentation.

Polymorpho and mononuclear leucocytes were almost completely separated by this purification process. In the mononuclear leucocyte fraction, the percentage of lymphocytes and monocytes was 95 ± 3.6 and 3.6 ± 2.6% respectively.

TABLE I

DIFFERENTIAL COUNTS OF LEUCOCYTE PREPARATIONS AFTER SEPARATION WITH FICOLL-ISOPAQUE SOLUTION OR SINGLE DEXTRAN SOLUTION

The data represent mean percent values ± standard deviation from 5 preparations. Each time 100 cells were counted.

Ficoll-Isopaque solution	Neutrophil	Eosinophil	Basophil	Mononuclear
Mononuclear fraction ($2.4 \cdot 10^7$) *	1.6 ± 3.5	— —	0.3 ± 0.5	98.4 ± 3.6
Polymorphonuclear leucocyte fraction ($6.5 \cdot 10^7$) *	93 ± 3.1	6.0 ± 2.6	— —	1.3 ± 1.0
Single Dextran fraction ($9.9 \cdot 10^7$) *	82 ± 7.3	2.0 ± 1.4	1.6 ± 1.4	14 ± 7.8

* Total cell counts per 100 ml of blood.

When isolating leucocytes by dextran only, about 50% more cells were obtained, but with a proportion of 14% mononuclear leucocytes.

Effects of pH on proteolytic activity of polymorpho and mononuclear leucocytes

The activities of proteases in homogenates of granule fractions, over the pH range from 2.7 to 8.0, are shown in Fig. 1.

In polymorphonuclear leucocytes two peaks of protease activities were

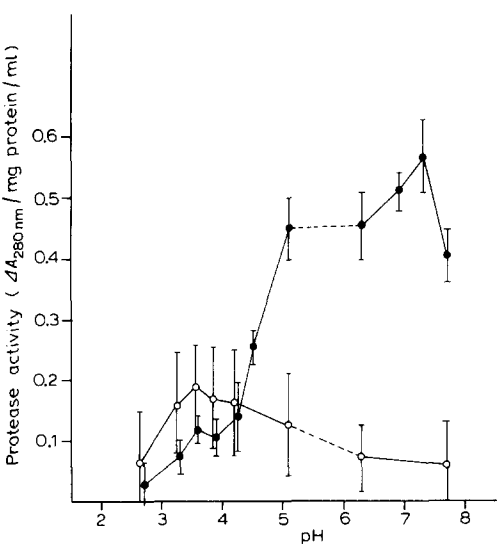

Fig. 1. pH activity profiles of proteases from human leucocytes. Polymorphonuclear leucocytes, (●); mononuclear leucocytes, (○). Five experiments were performed with the granules of polymorphonuclear leucocytes and six with mononuclear leucocytes. Vertical bars show the standard deviations.

found, one at pH 3.6 (acid protease) and the second at pH 7.3 (neutral protease). In the acid range, the difference of activity between pH 3.6 and 4.2 was not significant at the 0.05 level of probability. As indicated by the dotted part of the graph in Fig. 1, different results were found between pH 5 and 6 when using different buffers: at pH 5.5 the difference of extinction at 280 nm was indeed much higher in citric acid buffer than in phosphate buffer, in spite of identical blank values.

The activity of neutral protease was several times higher than that of the acid enzyme both in the whole cell homogenate (3 times) and in the granular fraction (5 times). As one could expect, the enzyme specific activities of the granular fraction were much higher than those found in the homogenates of whole cells, this being particularly evident at pH 7.3.

It is important to observe that in mononuclear leucocytes only one peak of protease activity was found (pH 3.6) with a specific activity much higher than in polymorphonuclear leucocytes.

Intracellular distribution of enzyme activity

As expected, most of the activity of the three enzymes, acid protease, neutral protease and β-glucuronidase was found in the granule fraction (Table II). The percentage distribution of neutral protease was identical to that of β-glucuronidase. As for acid protease, a significant portion of enzyme activity (22%) was also found in the nuclear fraction. Interference by nucleic acids can be ruled out since the measurements were performed at 750 nm.

Extraction of proteases from polymorphonuclear leucocytes granules for chromatography

The procedure of freezing and thawing, utilised in the early part of our work, was found to be very efficient for the liberation of acid protease but not of the neutral enzyme. After freezing and thawing the granular fraction ten times, 81% of the acid protease activity, and only 34% of the neutral enzyme, were liberated (as compared to the activity measured when using Triton X-100).

For the liberation of the neutral protease, 0.1% Triton X-100 was therefore used. However, when applying the enzyme solution containing the detergent on

TABLE II

DISTRIBUTION OF ENZYMES AND PROTEIN IN SUBCELLULAR FRACTIONS OF POLYMORPHONUCLEAR LEUCOCYTES

The enzyme activities were determined in defreezed aliquots of the various fractions, stored in the presence of Triton X-100, as explained in Materials and Methods.

Enzymes	Subcellular fraction		
	Nuclear (%)	Granule (%)	Supernate (%)
Acid protease (pH 3.6)	22.0	63.9	14.1
Neutral protease (pH 7.3)	3.0	84.4	12.6
β-glucuronidase (pH 5.0)	2.2	84.9	13.0
Soluble protein	9.9	19.3	70.9

the column, much of the white material did not migrate, even when it had been dialyzed for 48 h. Therefore the enzyme had to be sedimented by means of acetone before chromatography, as explained in Materials and Methods, in order to eliminate Triton X-100 and precipitate the proteins. These were then made soluble by adding 10 mM EDTA 2 Na in Tris · HCl buffer. After elimination of EDTA, a satisfactory elution of neutral protease by ion exchange chromatography could then be obtained.

One has to add that EDTA, at the concentration used in the present experiment, had no inhibitory effect upon either the acid or the neutral enzymes.

DEAE chromatography of the granule fraction from pure polymorphonuclear leucocytes, after extraction by freezing and thawing

Extracts prepared by freezing and thawing the granule fraction of pure polymorphonuclear leucocytes were subjected to DEAE chromatography.

The first peak obtained on elution was made up of cationic proteins including neutral protease; it was followed by a small second component. After application of 0.1 M NaCl to the column, a third component appeared, made up of anionic proteins, and containing both the peaks of acid protease and β-glucuronidase. The amount of neutral protease (first peak) was much lower than that of acid protease. This difference can be explaiend on the basis of what was just said on the effect of freezing and thawing upon the extraction of the enzymes. Some proteins were still eluted after application of 0.5 M NaCl, but no proteolytic activity could be found in these fractions.

Attempts to elute the whole of the acid protease by 0.05 M NaCl were not successful.

When, instead of stepwise gradients, a continuous NaCl gradient, up to

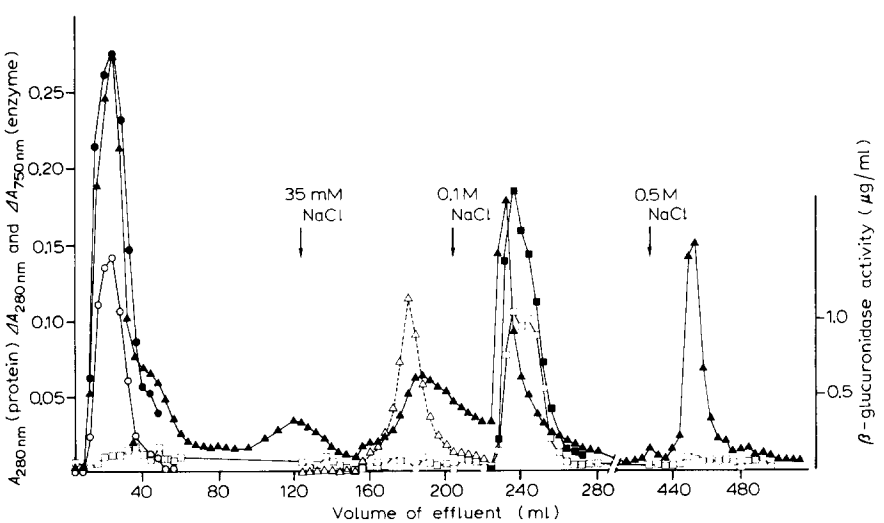

Fig. 2. Chromatography of granule fraction from whole leucocytes. DEAE Sephadex A 50 (1.6 cm × 20 cm bed) was equilibrated with Tris · HCl buffer (50 mM, pH 7.8). Protein (▲); neutral protease at 280 nm (○); neutral protease at 750 nm (●); acid protease at 280 nm (□) and acid protease at 750 nm (■). Flow rate 18 ml/h, collected in 4-ml fractions. 0.1 ml was used for enzyme assay and incubated for 3 h at 38°C with haemoglobin.

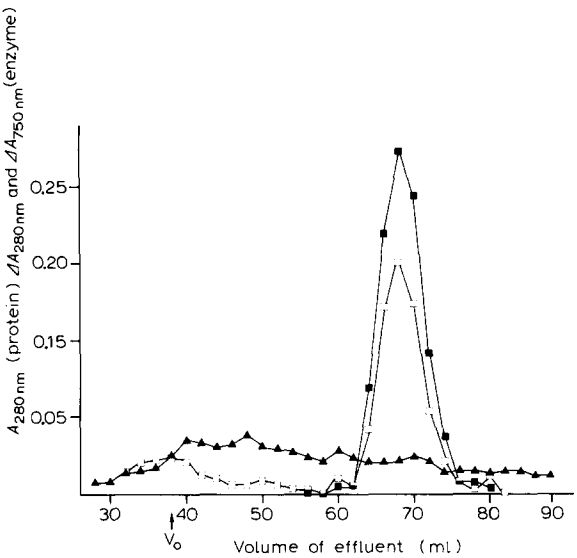

Fig. 3. Effluent concentration profiles for protein (▲); acid protease at 280 nm (□) and at 750 nm (■); when a sample of acid enzyme obtained by DEAE chromatography from whole leucocytes was passed through a column (1.6 cm X 70 cm) of Sephadex G-200 superfine. Flow rate 1.8 ml/h, collected in 2-ml fractions.

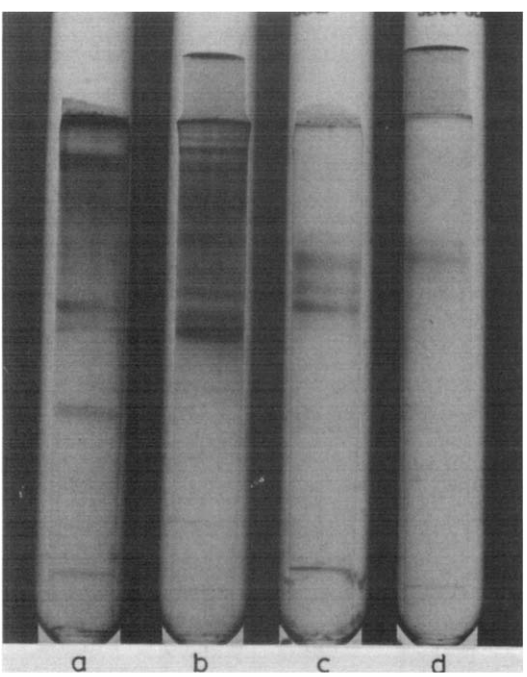

Fig. 4. Disc gel electrophoresis of (a) homogenate of pure polymorphonuclear leucocytes; (b) the acid protease peak from DEAE chromatography; (c) the acid protease peak from DEAE G-200 and (d) the peak from Sephadex G-200 superfine. The discontinuous Tris-glycine buffer system of Davis [18] was used and the current was 2 mA/tube. The band in the bottom area (anode) is tracing dye.

0.15 M, was used, similar peaks of β-glucuronidase and acid protease were obtained, the latter appearing between 0.038 and 0.11 M NaCl, with a maximum elution at 0.058 M.

The acid protease eluted by DEAE ion exchange chromatography showed a single peak of activity in the pH range 2.7—6.0 and an optimum pH at 3.6.

Further purification of cathepsin D from leucocytes

The acid protease separated from polymorphonuclear leucocytes by the process of DEAE chromatography had an optimum pH typical of cathepsin D.

Therefore, further purification was attempted in order to completely characterize the enzyme. Crude enzyme solution, extracted by acetone from granules of whole leucocytes was run through DEAE Sephadex A-50 (Fig. 2). This time a high yield of neutral protease was obtained as well as a complete separation of β-glucuronidase from the acid protease.

The acid protease fraction was then concentrated and run through Sephadex G-200; a peak of acid protease was obtained by this first gel chromatography. However, when run through disc gel electrophoresis three bands were found for this material (Fig. 4c).

Better results were obtained with Sephadex G-200 superfine. Concentrated enzyme solutions obtained after G-200 chromatography or directly after DEAE chromatography gave a sharp peak of enzyme activity, always in the same position, when passed through Sephadex G-200 superfine (Fig. 3). A single protein band was obtained this time (R_f = 0.31), with a trace of a more slowly moving component, when checking the purity by electrophoresis (Fig. 4d). The single band obtained with Sephadex G-200 superfine (Fig. 4d) corresponded to the highest of the three bands obtained by normal G-200 (Fig. 4c).

A summary of the process of purification is presented in Table III. A minute amount of protein was obtained after the passage over sephadex G-200 superfine, which explains the absence of a protein peak in the elution profile of Fig. 3. As shown in the last column of Table III, Cathepsin D was purified about 400 times with our procedure, when considering the activity in the pooled fractions making up the final chromatography peak.

Contrary to neutral protease and cathepsin BI, our purified cathepsin D was not inhibited by human serum.

The activity of the purified enzyme was measured as a function of time, using an estimated amount of 0.6 μg of protein in 0.1 ml. At 280 nm, linearity

TABLE III

PURIFICATION PROCESS OF CATHEPSIN D FROM HUMAN LEUCOCYTES

	Protein (mg)	Specific activity	Yield (%)	Purification factor
Homogenate	195.22	0.0335	100	1
Granule fraction	42.99	0.090	59	2.68
Acetone extract	6.98	0.31	33	9.25
DEAE chromatography	1.06	1.21	20	36.1
G-200 superfine	0.067	13.73	14	409.8

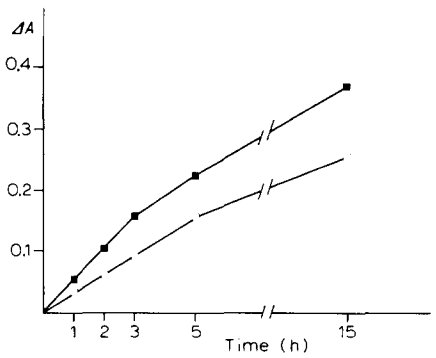

Fig. 5. Activity of the purified cathepsin D as a function of the time of incubation: at 280 nm (□); at 750 nm (■).

was observed up to 5 h incubation time. At 750 nm a linear relationship could be found up to 3 h (Fig. 5).

Estimation of molecular weight

By the gel chromatography on Sephadex G-200 superfine, blue dextran emerged with a peak at 38 ml (V_0). Bovine serum albumin, ovalbumin, chymotrypsinogen A and myoglobin emerged at 57.5, 65, 80 and 88.25 ml, respectively. The precise total bed volume (V_t), including both the liquid volume and the gel volume was of 108 ml. K_{av} for bovine serum albumin, ovalbumin, chymotrypsinogen A and myoglobin was 0.2785, 0.3857, 0.6000 and 0.7178, respectively.

These values gave a linear relation to their molecular weight. Cathepsin D was eluted at 68 ml and gave a value of 0.4285 for K_{av}, which corresponds to a molecular weight of 42 000.

Discussion

Cathepsin D is an intracellular acid protease widely distributed throughout many animal tissues [21]. It is one of the chief protein digesting enzymes in lysosomes, known to be present at high concentrations in inflamed tissues [23]. When released, Cathepsin D would be capable of attacking the protein-polysaccaride complexes of the surrounding matrix [24,25].

The enzyme has been purified from bovine spleen [26—29], rabbit spleen [30], rabbit liver [13,20], human liver [20], human erythrocytes [33] and human articular cartilage [34]. Concerning leucocytes, Cohn ad Hirsch [35], were able to demonstrate Cathepsin D among the many hydrolases found in the cytoplasmic granules, similar to lysosomes, isolated from rabbit polymorphonuclear leucocytes. The enzyme was also demonstrated in polymorphonuclear leucocytes from several other animals and was purified from rabbit and pig leucocytes [36,37].

In human leucocytes, the distribution of cathepsin D has been studied by several investigators [1,38—40]. In the granule fraction of human polymorphonuclear leucocytes it has repeatedly been reported that the activity of cathepsin

D is 3- to 10-times smaller when compared to that of the neutral protease. Our results confirm these observations and show that Cathepsin D activity in the granule fraction is 5-times smaller than that of neutral protease. In this context, one has to remember that the type of buffer and the nature of haemoglobin can influence the results at different pH values. Concerning the buffer, a difference of activity was for instance found at pH 5.5 when using citric acid buffer or phosphate buffer (Fig. 1). As for the haemoglobin, this molecule is known to reversibly dissociate into 2 subunits at pH 4.5 and is irreversibly degraded into 4 subunits near pH 3 [41]. Clearly these changes could have an influence upon the results of enzyme assays at different pH values.

In the assays of protease the final liberation of small peptides and free amino-acids from haemoglobin was measured either by the absorbance at 280 nm or by the absorbance at 750 nm after adding the Folin-Ciocalteau reagent. It is important to point out that the second method has given better results, with relatively low blank values. In most of our experiments, both methods were used.

As one can see in the pH profile shown in Fig. 1, which is similar to that published in 1968 by Janoff and Zeligs [6], the peak of acid protease is too small and does not allow any statement on the actual presence of cathepsin D in human polymorphonuclear leucocytes. A clear separation of the acid from the neutral enzyme could be obtained by ion exchange chromatography. Moreover, this technique allowed the additional separation of β-glucuronidase in the anionic protein component. No acid protease activity could be eluted at high salt concentrations (0.5 M NaCl) which probably means that cathepsin E (EC 3.4.4) is not present in human leucocytes.

Ion exchange chromatography was also used by Lazarus et al. [42] in their study of collagenase and by Janoff [43] for the separation of neutral protease from human polymorphonuclear leucocytes. In the elution profiles obtained by these investigators, neutral collagenase was shown in the anionic component eluted between 0.045 and 0.075 M NaCl, in the same region where we found the peak of cathepsin D activity.

Further purification of cathepsin D was obtained by gel chromatography. Attempts to purify the enzyme with either Sephadex G-75 or G-100, as often performed with extracts of other tissues [20,28—31], did not give a sufficient separation. This was probably due to two factors: the variety of anionic proteins present, as shown by electrophoresis, and the small amounts of protein, which forced us to use short columns. When using Sephadex G-200 superfine, in runs lasting as long as 48 h, the enzyme could be separated in relatively short columns.

The negative inhibition test with serum showed that the preparation did not contain cathepsin BI, an enzyme known to be inhibited by serum [44]. No other methods were used to identify the purity of our final preparation.

As for the molecular weight, the values reported in the literature for cathepsin D from other sources lie between 35 000 and 58 000. It is important to mention that the cathepsin D peak obtained with G-200 superfine was located only 3 ml following that of ovalbumin, similarly to what has been reported when using other types of chromatography gels [44].

Our results seem to leave little doubt that cathepsin D is present also in

human polymorphonuclear leucocytes and should stimulate further research into its biological significance.

Acknowledgements

We are very grateful to Mrs I. Condacci and Miss E. Andersen for their skillful technical assistance. We also thank Dr. M. Jeannet and Prof. P.A. Miescher for supplying human blood and Dr. M. Baggiolini, Privat Dozent, Wander, Bern, for helpful criticism and suggestions. This work was supported by grants 3.1660.73 and 3.604-075 of the Swiss National Fund for scientific research.

References

1 Weissman, G., Zurier, R.B. and Hoffstein, S. (1972) Am. J. Pathol. 68, 539—559
2 Weston, P.D., Barrett, A.J. and Dingle, J.T. (1969) Nature 222, 285—286
3 Morrison, R.I.G., Barrett, A.J., Dingle, J.T. and Prior, D. (1973) Biochim. Biophys. Acta 302, 411—419
4 Ishikawa, I., Cimasoni, G. and Ahmad-Zadeh, C. (1972) Arch. Oral Biol. 17, 111—117
5 Hasegawa, K., Cimasoni, G.and Vuagnat, P. (1975) Experientia 31, 765—766
6 Janoff, A. and Zeligs, J.D. (1968) Science 161, 702—704
7 Janoff, A. (1973) Lab. Invest. 29, 458—464
8 Ohlsson, K. and Olsson, I. (1974) Eur. J. Biochem. 42, 519—527
9 Dewald, B., Rindler-Ludwig, R., Bretz, U. and Baggiolini, M. (1975) J. Exp. Med. 141, 709—723
10 Folds, J.D., Welsh, I.R.H. and Spitznagel, J.K. (1972) Proc. Soc. Exp. Biol. Med. 139, 461—463
11 Böyum, A. (1967) Scand. J. Clin. Lab. Invest. 21 (Suppl. 97), 77—89
12 Fallon, H.J., Frei, E., Davidson, J.D., Trier, J.S. and Burk, D. (1962) J. Lab. Clin. Med. 59, 779—791
13 Barrett, A.J. (1970) Biochem. J. 117, 601—607
14 Anson, M.L. (1938) J. Gen. Physiol. 22, 79—89
15 Mead, J.A.R., Smith, J.N. and Williams, R.T. (1955) Biochem. J. 61, 569—574
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
17 Dawson, R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M. (1969) Data for Biochemical Research 2nd edn., pp. 625—626
18 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
19 Chrambach, A., Reisfeld, R.A., Wyckoff, M. and Zaccari, J. (1967) Anal. Biochem. 20, 150—154
20 Barrett, A.J. (1967) Biochem. J. 104, 601—608
21 Kuchler, R.J. (1972) in Manometric and Biochemical Techniques (Umbreit, W.W., Burris, R.H. and Stauffer, J.F. eds.), 5th edn., pp. 331—341, Burgess Publishing Co., Minneapolis
22 Barrett, A.J. (1972) in Lysosomes A Laboratory Handbook (Dingle, J.T. ed.), pp. 95—100, North-Holland Publishing Co., Amsterdam
23 Bazin, S. and Delaunay, A. (1970) in Inflammation Biochemistry and Drug Interaction (Bertelli, A. Houck, J.C. eds.), pp. 21, Excerpta Media, Amsterdam
24 Dingle, J.T., Barrett, A.J. and Weston, P.D. (1971) Biochem. J. 123, 1—13
25 Woessner, J.F. (1971) in Tissue Proteases (Barrett, A.J. and Dingle, J.T. eds.), pp. 291—308, North-Holland Publishing Co., Amsterdam
26 Press, E.M., Porter, R.R. and Cebra, J. (1960) Biochem. J. 74, 501—514
27 Keilova, H. (1971) in Tissue Proteases (Barrett, A.J. and Dingle, J.T. eds.), pp. 45—65, North-Holland Publishing Co., Amsterdam
28 Ferguson, J.B., Andrews, J.R., Voynick, I.M. and Fruton, J.S. (1973) J. Biol. Chem. 19, 6701—6708
29 Smith, R. and Turk, V. (1974) Eur. J. Biochem. 48, 245—254
30 Lapresle, C. and Webb, T. (1960) Biochem. J. 76, 538—543
31 Woessner, J.F. and Schamberger, R.J. (1971) J. Biol. Chem. 246, 1951—1960
32 Schwabe, C. and Kalnitsky, G. (1965) Arch. Biochem. Biophys. 109, 68—75
33 Reichet, D., Jacobson, E. and Haschen, R.J. (1974) Biochim. Biophys. Acta 341, 15—26
34 Sapolsky, A.I., Howell, D.S. and Woessner, J.F. (1974) J. Clin. Invest. 53, 1044—1053
35 Cohn, Z.A. and Hirsch, J.G. (1960) J. Exp. Med. 12, 983—1004
36 Lapresle, C. (1971) in Tissue Proteases (Barrett, A.J. and Dingle, J.T. eds.), pp. 135—150, North-Holland Publishing Co., Amsterdam
37 Kopitar, M., Kregar, I. and Lebez, D. (1971) Enzymiologia 41, 129—139
38 Haschen, R.J. and Krug, K. (1966) Nature 209, 511—512

39 Fraenkel-Conrat, J., Chew, W.B., Pitlick, F. and Barber, S. (1966) Cancer 19, 1393—1398
40 Stiles, M.A. and Fraenkel-Conrat, J. (1968) Blood 32, 119—133
41 Rossi Fanelli, A., Antonini, E. and Caputo, A. (1964) Advan. Protein Chem. 19, 73—222
42 Lazarus, G.S., Daniels, J.R., Brown, R.S., Bladen, H. and Fullmer, H.M. (1968) J. Clin. Invest. 47, 2622—2629
43 Janoff, A. (1970) Lab. Invest. 22, 228—235
44 Starkey, Ph. and Barrett, A.J. (1973) Biochem. J. 131, 823—831
45 Schwabe, C. (1975) J. Dent. Res. 54, 371—377