Collagenolytic Cathepsin Activity in Rabbit Peritoneal Polymorphonuclear Leucocyte Granules

By WALTER T. GIBSON,*† DAVID W. MILSOM,* FRANK S. STEVEN* AND JOHN S. LOWE‡

*Department of Medical Biochemistry, University of Manchester, Oxford Road, Manchester M13 9PT, U.K.,
and ‡Department of Biochemistry, I.C.I. Ltd. Pharmaceuticals Division, Alderley Park, Macclesfield,
Cheshire, U.K.

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Collagenolytic cathepsin activity was detected in lysed rabbit peritoneal polymorphonuclear leucocytes. The pH optimum was around 3, and activity was greatly enhanced by the presence of cysteine and EDTA. Digestion of polymeric collagen resulted in the release of α -, β - and γ -chains. Collagenolytic cathepsin activity was associated mainly with the granule fraction isolated from homogenates by differential centrifugation. The granule fraction was further fractionated by isopycnic density-gradient centrifugation, and the collagenolytic cathepsin activity was shown to be associated with the azurophil and tertiary granules, both lysosome-like organelles.

Collagenolytic cathepsins have been detected in a variety of mammalian tissues, including rat granulation tissue (Bazin & Delaunay, 1971), rat post-partum uterus (Etherington, 1973) and bovine spleen (Etherington, 1976). They are characterized by a low pH optimum, activation by thiol compounds, and the ability to degrade insoluble polymerized collagen. Although cathepsin B may be responsible for some of the above examples of collagenolytic activity (Burleigh *et al.*, 1974), it has been shown, in bovine spleen, that the collagenolytic cathepsin is distinct from cathepsin B (Etherington, 1976).

Although the accumulation of rabbit polymorphonuclear leucocytes at the site of an inflammatory response is usually associated with tissue damage (Henson & Cochrane, 1975), there has been no previous report of a collagenolytic cathepsin in these cells. We have obtained evidence for the presence and subcellular location of collagenolytic cathepsin activity in rabbit polymorphonuclear leucocytes. Preliminary reports of this work have been published (Gibson *et al.*, 1976a,b).

Experimental

Materials

Guaiacol (*o*-methoxyphenol) and 4-nitrophenyl disodium orthophosphate were purchased from BDH Chemicals, Speke, Liverpool, U.K. 4-Methylumbelliferyl 2-acetamido- β -D-glucopyranoside was bought from Koch-Light Laboratories, Colnbrook,

Bucks., U.K. Bovine achilles tendons were kindly supplied by Manchester Abattoir. Type-I tropo-collagen from guinea-pig skin was a gift from Dr. C. A. Shuttleworth, Department of Medical Biochemistry, University of Manchester. Acid-soluble collagen was extracted and purified from calf skin or rat tail tendon by the method of Steven & Jackson (1967). Chemicals were of analytical grade whenever possible.

Methods

Collection of cells. Glycogen-induced peritoneal-exudate polymorphonuclear leucocytes (here after referred to as leucocytes) were obtained from adult rabbits by the method of Cohn & Hirsch (1960), except that a longer period (16 h rather than 4 h) was left between injection of the glycogen solution and collection of the exudate. The cells were collected in 0.9% NaCl containing 50 i.u. of heparin/ml to prevent agglutination. Exudates that were contaminated with blood were discarded. Exudates were pooled and centrifuged at 980 g_{av} for 10 min at 4°C. The cells were washed by resuspending in fresh NaCl/heparin and re-centrifugation under the same conditions. The average yield of cells was 7.9×10^8 per rabbit. Over 90% of the cells in the exudate were neutrophil polymorphonuclear leucocytes as shown by differential counts. The remainder were mainly erythrocytes, macrophages and eosinophils.

Preparation of polymeric collagen. This was isolated from bovine achilles tendon by the EDTA method of Steven (1967), with the following modifications. The disintegration of frozen tendons was omitted, and homogenization of tendon pieces was replaced by vigorous stirring overnight in 0.2M-acetic acid at

† To whom reprint requests should be addressed at his present address: Environmental Safety Division, Unilever Research, Colworth House, Sharnbrook, Bedford MK44 1LQ, U.K.

4°C. After precipitation, polymeric collagen fibrils were allowed to equilibrate at neutral pH and were then washed thoroughly with water and freeze-dried.

Assays. Collagenolytic cathepsin activity was measured by the solubilization of hydroxyproline-containing material from 5mg of polymeric collagen at 37°C and pH 4.0. Digested material was separated from the residue by centrifugation at 980g_{av.} for 10min and samples were hydrolysed at 100–110°C in 6M-HCl for 18h. Thereafter, HCl was removed by rotary evaporation and the hydroxyproline content of each hydrolysate determined by the method of Woessner (1961). Details of the composition of assay mixtures are given in the legends. Activity is expressed as μg of collagen solubilized/min. The amount of polymeric collagen digested was proportional to the time of incubation, and the rate of digestion was proportional to the amount of enzyme source added.

In isopycnic density-gradient fractionation experiments the collagenolytic cathepsin assay was modified as follows. Assay mixtures contained 2.5mg of polymeric collagen, 0.5ml of 0.2M-sodium acetate buffer, pH 4.0, 0.8ml of fraction and final concentrations of 10mM-L-cysteine, 1mM-EDTA and 0.1% Triton X-100 (final volume 1.5ml). The pH of the final reaction mixture was checked and found to be 4.0. Controls contained 0.8ml of 2.45M-sucrose instead of fraction. After incubation at 36°C for 18h, each assay mixture was diluted to 3ml with 0.2M-sodium acetate buffer, pH 4.0. After centrifugation at 980g_{av.} for 10min two 1ml samples of the supernatant were taken from each tube. These were heated to 60°C for 1h to denature collagenous material, diluted to 2.5ml, adjusted to pH 2.0 with 0.1M-HCl and subjected to resin-catalysed hydrolysis. This was performed by mixing with 1g of Dowex 50W resin (X8; H⁺ form, 20–50 U.S. mesh), and then washing off non-ionic material (sucrose) with 3 × 5ml of water. The samples were then incubated at 105°C for 18h in sealed tubes with the level of liquid just above the top of the resin. Thereafter the resin was eluted with 3 × 5ml of 1.55M-NH₃ and the hydroxyproline content of the eluates determined by the method of Woessner (1961).

Cathepsin B (EC 3.4.22.1) activity was assayed by the hydrolysis of α -N-benzoyl-L-arginine ethyl ester in a pH-stat (Radiometer, from V. A. Howe, London SW6, U.K.) connected to an auto-burette which titrated the acid as it was released. Assay mixtures contained 20mM- α -N-benzoyl-L-arginine ethyl ester, 0.3mM-L-cysteine, 0.3mM-EDTA and enzyme sample, made up to a final volume of 3ml with 0.001M-sodium acetate buffer, pH 4.0. Incubation was at 37°C. Trypsin (Armour Pharmaceutical, Hampden Park, Eastbourne, Sussex, U.K.) activity against the same substrate was measured at pH 7.9.

N-Acetyl- β -glucosaminidase (EC 3.2.1.30) was assayed by the fluorimetric technique of Barrett (1972),

with 4-methyl-2-acetamido-2-deoxy- β -D-glucopyranoside (Koch-Light Laboratories) as substrate, at pH 4.3.

Arylsulphatases A and B ('arylsulphatase', EC 3.1.6.1) was assayed as follows: 0.2ml of enzyme sample was mixed with 0.2ml of substrate solution (0.02M-dipotassium nitrocatechol sulphate in 1M-sodium acetate buffer, pH 5.5, containing 0.2% Triton X-100), and incubated at 37°C for up to 0.5h. After stopping the reaction with 3ml of 0.2M-NaOH, the amount of nitrocatechol released was determined by its A_{515} ($\epsilon \approx 11\,200 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$).

Acid *p*-nitrophenyl phosphatase (EC 3.1.3.2) was assayed as follows: 0.1 or 0.2ml of fraction was incubated with an equal volume of substrate solution containing 16mM-4-nitrophenyl disodium orthophosphate and 0.2% w/v Triton X-100 in 0.2M-sodium acetate buffer, pH 5.0, at 37°C for up to 15min. The reaction was stopped by the addition of 3.0ml of cold 0.2M-NaOH, and the 4-nitrophenol liberated was measured as A_{420} ($\epsilon \approx 18\,200 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$). Alkaline phosphatase (EC 3.1.3.1) assay mixtures contained 0.1ml of fraction and 0.9ml of substrate solution containing 10mM-4-nitrophenyl disodium orthophosphate and 0.1% (w/v) Triton X-100 in 0.1M-glycine/NaOH buffer, pH 9.8. Incubation was at 37°C for up to 15min and the reaction was stopped by adding 3.0ml of cold 0.2M-NaOH. 4-Nitrophenol released was measured as A_{420} ($\epsilon \approx 18\,200$). Peroxidase (EC 1.11.1.7) activity was measured against *o*-methoxyphenol by a modification of the method of McRipley & Sbarra (1967). A sample (0.1ml) of the fraction was mixed in a cuvette with 2.9ml of a solution of 6.7mM-*o*-methoxyphenol in 6.7mM-sodium phosphate buffer, pH 7.0, containing 0.1% Triton X-100, and placed in a recording spectrophotometer. The mixture was allowed to stabilize for about 1min, then 20 μl of 10mM-H₂O₂ was added rapidly and the initial rate of increase in A_{470} was measured.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V) as standard.

Enzyme sources. In most experiments the rabbit leucocyte-granule fraction, isolated by differential centrifugation, was used as the source of collagenolytic cathepsin activity. On some occasions a leucocyte lysate was used instead. The latter was prepared by sonicating a cell suspension (1×10^8 cells/ml in 0.9% NaCl) for 1min at a peak-to-peak (amplitude) setting of 2 on an MSE (Crawley, Sussex, U.K.) sonicator. This was followed by autolysis at pH 4.0 and 37°C for 1h, and the preparation was filtered before use. Leucocyte-granule fractions were prepared as described below.

Homogenization and differential centrifugation of rabbit leucocytes. Washed rabbit leucocytes were resuspended in ice-cold 0.34M-sucrose containing 50i.u.

of heparin/ml ('sucrose/heparin') to give a concentration of 3×10^7 cells/ml. Heparin was essential for efficient homogenization, which was performed by vigorously passing the suspension up and down through a Millipore filter-support grid (Swinnex type, Millipore, London N.W.10, U.K.) attached to a 50ml syringe. About five to ten strokes were adequate. The suspension was diluted with 2 vol. of sucrose/heparin to decrease the viscosity before centrifugation. The differential-centrifugation scheme was as follows. The homogenate was centrifuged for 10 min at $605g_{av}$ and 4°C in a Mistral 6L centrifuge (12×100 ml swing-out rotor). The pellet was resuspended in fresh sucrose/heparin (about one-fifth the original volume) and re-centrifuged under the same conditions. The pellet was retained and the first and second supernatants were combined and centrifuged at $11500g_{av}$ and 4°C for 15 min. The pellet was washed by resuspension and re-centrifugation as before, and the supernatants were combined. The first and second pellets (fractions I and II respectively) were resuspended in sucrose/heparin for assay. The final combined supernatant was referred to as fraction III. Assays were performed within 48 h of isolating these fractions, and the relative specific activity (percentage of total recovered enzyme activity in a given fraction/percentage of total recovered protein in that fraction) of each marker enzyme in each fraction was calculated.

Isopycnic-density-gradient centrifugation of a rabbit leucocyte post-nuclear supernatant. Rabbit leucocytes were collected, washed and homogenized as described above except that cells were suspended to a concentration of 1×10^8 /ml for homogenization. A post-nuclear supernatant was obtained by centrifuging the homogenate at $605g_{av}$ and 4°C for 10 min. This supernatant was adjusted to a concentration of $0.74M$ with respect to sucrose by adding $2.45M$ -sucrose, and 2 ml samples were applied to continuous linear gradients of 0.74 – $2.45M$ -sucrose in 16 ml, resting on a cushion of 2 ml of $2.45M$ -sucrose. Centrifugation was at 4°C in a 3×25 ml swing-out rotor (r_{av} 9.39 cm) for the integrated field-time of $5.6 \times 10^6 g\text{-min}$. Fractions (1 ml) were collected by displacement with $2.45M$ -sucrose.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. For this, 5% polyacrylamide gels were made by the method of Furthmayr & Timpl (1971). A current of 6 mA per tube was used, with a running time of 3 h. Gels were stained for 1 h in 0.25% (w/v) Coomassie Blue in 20% (w/v) trichloroacetic acid and destained in 7% (w/v) acetic acid.

Results and Discussion

Properties of rabbit leucocyte collagenolytic cathepsin

Whole lysates of rabbit leucocytes were capable of degrading polymeric collagen at pH 4.0 and 37°C , and

the specific activity of this enzyme source was calculated to be $0.24 \mu\text{g}$ of collagen solubilized/min per mg of protein. No cathepsin B activity could be detected in similar preparations of rabbit leucocytes by the titrimetric technique used. This is in agreement with the results of Wasi *et al.* (1966), who also failed to detect cathepsin B activity in these cells. Therefore the collagenolytic activity was termed 'collagenolytic cathepsin' by analogy with the enzyme in bovine spleen which Etherington (1976) has shown to be separable from cathepsin B.

Collagenolytic cathepsin activity was maximal at pH 3 (Fig. 1), which is close to the pH optima of collagenolytic cathepsins from other sources (Milsom *et al.*, 1972; Etherington, 1972). To minimize the risk of denaturing the substrate, however, routine assays of collagenolytic cathepsin were performed at pH 4.0. At this pH and 37°C , polymeric collagen is much more stable than tropocollagen and underwent no increase in susceptibility to trypsin after 18 h incubation under these conditions.

In common with collagenolytic cathepsins from human and rat liver (Milsom *et al.*, 1972; Etherington, 1972), the enzyme from rabbit leucocytes was activated by cysteine and EDTA (Fig. 2). In their

Fig. 1. Effect of pH on collagenolytic activity

Each assay contained 5 mg of polymeric collagen, 1 ml of $0.2M$ -sodium citrate buffer adjusted to the appropriate pH with NaOH, 0.3 ml of rabbit leucocyte-granule fraction and 0.1% (w/v) Triton X-100, 10 mM-L-cysteine and 10 mM-EDTA (final concentrations) in a total volume of 1.5 ml. Incubation was at 36°C for 4 h. Control values have been subtracted.

Fig. 2. Effect of cysteine and EDTA on collagenolytic cathepsin activity

Assay mixtures were as described in the legend to Fig. 1, except that the pH was maintained at 4.0 and cysteine and/or EDTA were added to give final concentrations of 1 or 10 mM. Incubation was at 36°C for 3 h. Control values have been subtracted.

absence, activity was low and variable. Cysteine alone produced a large increase in activity, but EDTA alone gave only a slight enhancement. However, the combination of both cysteine and EDTA caused a marked activation of the collagenolytic cathepsin, which was much more than the sum of the individual effects (Fig. 2). The effect of EDTA seems therefore to be to enhance the activation by cysteine, and in this respect the rabbit leucocyte collagenolytic cathepsin is similar to papain (Smith & Kimmel, 1960). However, this is not sufficient evidence for the presence of an essential thiol group in the collagenolytic cathepsin. Inhibition studies designed to test this were unsuccessful owing to low activity in the absence of activators.

The solubilization of polymeric collagen involved the release of high-molecular-weight tropocollagen-like fragments. This is shown in Fig. 3, in which the electrophoretic mobilities of the digestion products, a tropocollagen standard and a control are compared. Bands corresponding to the α -, β - and γ -chains of the tropocollagen standard (gel a) were detected in the gel to which digestion products had been applied (gel b). No evidence was obtained for the presence of any material smaller than α -chains in the digest. The remaining bands on gel (b) correspond to those in the control (gel c) and thus arise from the enzyme source. The release of α - and β - as well as

Fig. 3. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of collagenolytic cathepsin digestion products. Polymeric collagen was digested at pH 4.0 and 37°C for 18 h with a homogenate of rabbit leucocytes. The digest supernatants were isolated by filtration through Whatman no. 1 paper. The control was incubated under the same conditions, but in the absence of the homogenate; a sample of the homogenate was added to the control supernatant immediately before electrophoresis. All samples were denatured in sample buffer before electrophoresis by heating to 100°C for 2 min. Gel (a), 20 μ l of a 4 mg/ml solution of neutral-salt-soluble guinea-pig skin collagen; gel (b), 50 μ l of digest supernatant; gel (c), 50 μ l of control supernatant. The positions of the α -, β - and γ -chains of tropocollagen are indicated.

γ -chains suggests that the enzyme cleaves the non-helical telopeptide extension, removing inter- and intra-molecular cross-linking regions.

Association of collagenolytic cathepsin with rabbit leucocyte granules

The granules of rabbit peritoneal leucocytes sedimented predominantly in fraction II of the differential-centrifugation scheme on the basis of the distribution profiles (from separate experiments) of the marker enzymes arylsulphatase and *N*-acetyl- β -glucosaminidase (Fig. 4). Both of these are granule enzymes, and fraction II contained the highest relative specific activity of each. In other terms, 50–60% of the total activity was recovered in this fraction. The relative specific activity of *N*-acetyl- β -glucos-

Fig. 4. Subcellular fractionation of rabbit leucocytes. The distribution profiles of (a) arylsulphatase, (b) collagenolytic cathepsin and (c) *N*-acetyl- β -glucosaminidase are shown. Collagenolytic cathepsin assay mixtures contained 1 ml of fraction, 0.9 ml of 0.2M-sodium acetate buffer, pH 4.0, and final concentrations of 1 mM-L-cysteine, 1 mM-EDTA and 0.1% (w/v) Triton X-100 in a total volume of 2.0 ml. Recoveries of enzyme activity were 80% for arylsulphatase, 95% for collagenolytic cathepsin and 84% for *N*-acetyl- β -glucosaminidase.

aminidase in fraction II was lower than that of arylsulphatase, and that of fraction III was correspondingly higher. In both cases most of the remaining activity was found in fraction III, which was considered to be the cytosol fraction and which also contained most of the recovered protein (60–80%). The amount of acid hydrolase activity found in this fraction was rather larger than that observed by Cohn & Hirsch (1960), and it may be that our use of heparin,

to lower the resistance of the plasma membrane to disruption, caused increased granule lysis. Only small amounts of arylsulphatase and *N*-acetyl- β -glucosaminidase were found in fraction I, which was observed in the optical microscope to consist mainly of intact leucocytes, erythrocytes and debris. The amount of protein and enzyme activity in this fraction probably depends on the efficiency of the homogenization, and varied slightly from experiment to experiment, as shown by the differences in protein distribution between Figs. 4(a) and 4(c).

The distribution profile of collagenolytic cathepsin activity (Fig. 4b) was similar to those of the granule marker enzymes, with the highest relative specific activity in fraction II. It was concluded that collagenolytic cathepsin is also of granule origin.

The granule fraction of rabbit leucocytes consists of several types of granule, which differ in size, density and enzyme content (Baggiolini *et al.*, 1970). The largest (azurophil) and the smallest (tertiary) are lysosome-like in their enzyme content, whereas the remaining class (specific) is intermediate in size and density, and is considered to be non-lysosomal. To determine the localization of collagenolytic cathepsin, the leucocyte post-nuclear fraction was subfractionated by isopycnic density-gradient centrifugation and the subfractions were assayed for their enzyme content.

Assay of collagenolytic cathepsin in density-gradient fractions

The presence of sucrose interfered with the assay of collagenolytic cathepsin activity in density-gradient fractions, causing a concentration-dependent loss of hydroxyproline. The losses occurred during the acid-hydrolysis stage of the assay, possibly as a result of humin formation (Block, 1960), and resulted in apparently low and variable distributions of activity. To surmount this problem an alternative assay method was developed in which acid hydrolysis was replaced by resin-catalysed hydrolysis. Preliminary experiments using acid-soluble collagen showed that resin hydrolysis by this method was much more effective on collagen which was heat-denatured before binding to the resin. Under these conditions the resin hydrolysis of denatured collagen was consistently about 80% as efficient as acid hydrolysis in the absence of sucrose. The resin method proved adequate as a routine procedure in the assay of large numbers of samples containing high sucrose concentrations, when conventional acid hydrolysis could not be used.

Localization of rabbit collagenolytic cathepsin activity in leucocyte granules

The distributions of marker enzymes for the three types of granule in the density gradient are shown in

Fig. 5. Distribution of collagenolytic cathepsin, marker enzymes and protein in subcellular fractions isolated from rabbit leucocytes by sucrose-density-gradient centrifugation

Details of the isolation procedure are given under 'Methods'. (a) Protein; (b) sucrose gradient; (c) peroxidase; (d) acid *p*-nitrophenyl phosphatase; (e) alkaline phosphatase; (f) collagenolytic cathepsin; (g) *N*-acetyl- β -glucosaminidase. The four main peaks of activity are labelled A, B, C and D in order of decreasing density.

Fig. 5. The enzyme data are expressed as relative activity, with the maximum for each enzyme adjusted to 100 for comparison. These results are in almost complete agreement with those of Baggiolini *et al.* (1970). The azurophil granules (fraction A) are marked by sharp peaks of peroxidase (Fig. 5c) and *N*-acetyl- β -glucosaminidase (Fig. 5g) at an equilib-

rium density of 1.26 g/ml. The sharp peak of alkaline phosphatase activity (Fig. 5e) at a density of 1.23 g/ml indicates the position of the specific granules (fraction B), and the second broader peak of *N*-acetyl- β -glucosaminidase at a density of 1.2 g/ml represents the tertiary granules (fraction C). Unlike Baggiolini *et al.* (1970), we observed a considerable overlap

between the large peak of protein (Fig. 5a) and that of acid *p*-nitrophenyl phosphatase (Fig. 5d), both of which equilibrate at a density of about 1.11 g/ml. This fraction (fraction D) contains mostly cytosol proteins, although some membranous material was detected by electron microscopy. Morphological observations of the fractions by electron microscopy were in general agreement with those of Baggiolini *et al.* (1970) and are not presented.

The distribution of collagenolytic cathepsin activity in the fractions is shown in Fig. 5(f). It is clearly similar to that of *N*-acetyl- β -glucosaminidase and shows two major peaks of activity associated with the azurophil and tertiary granules. Thus collagenolytic cathepsin activity is concluded to be localized in the two lysosome-like granule types of rabbit leucocytes. It is not possible from our data to determine whether the collagenolytic cathepsin activity is due to a single enzyme with a bimodal distribution or whether two distinct enzymes exist, each located in a different granule type.

It is concluded from the results in the present paper that rabbit peritoneal polymorphonuclear leucocytes contain a collagenolytic cathepsin with similar properties to collagenolytic cathepsins from other sources, and that it is located in the azurophil and tertiary granules of these cells.

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