Lysosomal Acid Proteinase of Rabbit Liver

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1. Acid proteinase from rabbit liver lysosomes was purified about 1000-fold, on a protein basis. 2. The purification procedure involved isolation of a lysosomal–mitochondrial pellet and conversion of this into an acetone-dried powder. 3. The enzyme was extracted with an acidic buffer and subjected to column chromatography with DEAE-Sephadex and Sephadex G-100. 4. The molecular weight of the enzyme was 50000–52000. 5. Maximal activity against haemoglobin was obtained at pH 3·2; serum albumin was attacked, but very much more slowly. 6. Several possible inhibitors of the enzyme were tested. Thiol-blocking reagents, several inhibitors of trypsin and chymotrypsin, and a chelating agent were without effect. 7. The enzyme was competitively inhibited by 3-phenylpyruvic acid at low concentrations. 8. Dithiothreitol caused rapid inactivation of the enzyme at pH 8. 9. It is concluded that this enzyme is a form of cathepsin D, which may be widely distributed in lysosomes.

There is good reason to believe that the acid proteinase, or 'cathepsin', of lysosomes plays an important role in cell physiology; it has been implicated in the pathological breakdown of cartilage matrix (Fell & Dingle, 1963; Ali, 1964) and in the degradation of muscle proteins in muscular dystrophy (Srivastava & Berlinguet, 1966; Weinstock, 1966). Other workers have suggested that the enzyme is concerned with collagen loss in involution of the uterus *post partum* (Woessner, 1965*a*,*b*), in regression of experimental granulomas (Robert, Cambier & Robert, 1965), the digestion of immunoprecipitates (Wasi, Uriuhara, Taichman, Murray & Movat, 1966) and bacteria by various leucocytes and in the formation of thyroid hormones (McQuillan, Mathews & Trikojus, 1961).

Several tissue acid proteinases have already been studied, but the enzyme of liver, in some ways the classical example of a lysosomal cathepsin (de Duve, Wattiaux & Baudhuin, 1962), seems to have received little attention. In the present paper the partial purification of the rabbit liver acid proteinase is described, and the properties of the enzyme are compared with those of acid proteinases from other sources. A preliminary report of part of this work has been published (Barrett, 1966).

MATERIALS

Rabbit livers from domesticated animals were kindly supplied by the Poultry Packing Station of J. Sainsbury Ltd. Tris (Trizma base), 2-oxoisohexanoic acid (α-oxoisocaproic acid), 3-phenylpyruvic acid, 3-(*p*-hydroxy)phenylpyruvic acid, crystalline bovine serum albumin, α-chymotrypsinogen A (type II), soybean trypsin inhibitor (type 1-S), pepsin (twice-crystallized, 2500–3200 units/mg.) and α-*N*-benzoyl-DL-arginine–*p*-nitroaniline HCl were obtained from Sigma (London) Chemical Co. Ltd. Sephadex and Blue Dextran 2000 were purchased from Pharmacia Fine Chemicals, and egg albumin (five times crystallized) from the Nutritional Biochemicals Corp. Trasylol (trypsin-kallikrein inhibitor from bovine parotid gland) was given by FBA Pharmaceuticals Ltd., DEAE-cellulose was Whatman DE-11, and dithiothreitol, phenylmethanesulphonyl fluoride and L-1-tosylamido-2-phenylethyl chloromethyl ketone were obtained from Calbiochem Ltd. Kodak Ltd. supplied 2,4′-dibromoacetophenone and 2-bromo-2-phenylacetophenone, and indolyl-3-pyruvic acid was purchased from Aldrich Chemical Co. Inc. Bovine haemoglobin was obtained from Armour Pharmaceutical Co. Ltd., and Triton X-100 from Lennig Chemicals Ltd.

The oxidized form of dithiothreitol was prepared by bubbling air through an aqueous solution until no further increase in E_{280} was obtained.

METHODS

Acid proteinase assay. A modification of Anson's (1939*a*) procedure was used. Bovine haemoglobin was suspended in water and dialysed at 4° for 24hr. The solution was then diluted to 8% (w/v) and frozen until required.

Incubation mixtures (1ml., pH 3·2) contained: sodium formate buffer (pH 3·0, 1·0M), 0·25ml.; enzyme sample, 0·5ml.; haemoglobin solution (8%, w/v), 0·25ml. After 1hr. at 45° the reaction was stopped by the addition of 5ml. of 3% (w/v) trichloroacetic acid from an automatic pipette. The contents of the tubes were mixed with a Whirlimixer (Scientific Industries Ltd.), incubated for a further 10min. and filtered; the E_{280} of the filtrate was measured. Reagent

blank values were obtained by adding the enzyme samples after the incubation period, immediately before the trichloracetic acid. Assays were carried out in duplicate.

One unit of enzyme activity was the quantity that would have produced an increase in extinction (ΔE_{280}) of 1·0 unit in the assay, a linear response being assumed. In practice the relationship between enzyme concentration and ΔE_{280} was linear only up to 0·3 unit, and usually assays were not performed with quantities of enzyme greater than this.

Three of the inhibitors tested, 3-phenylpyruvic acid, 3-(*p*-hydroxy)phenylpyruvic acid and indolyl-3-pyruvic acid, contributed excessively high extinctions in the standard assay procedure. A modified method was used therefore in which each trichloroacetic acid filtrate was shaken with ethyl acetate (2 ml.) and separated by centrifugation before the E_{280} was measured in the aqueous phase.

Acid phosphatase assay. This enzyme was measured by the method of Torriani (1960).

Activator and inhibitor tests. Various substances were included in the standard assay mixture to determine their effect on the enzyme activity. When necessary, e.g. with 6-aminohexanoic acid, the pH was adjusted with HCl. *p*-Choromercuribenzoic acid was dissolved in N-NaOH before use, and may have been hydrolysed to *p*-hydroxymercuribenzoate.

To test phenylmethanesulphonyl fluoride and L-1-tosylamido-2-phenylethyl chloromethyl ketone, stock solutions were prepared in methanol, so that all assay mixtures contained 3% (v/v) methanol. The buffered assay mixtures were stored for 2 hr. at 4° before the addition of substrate, to allow time for any interaction between enzyme and inhibitor.

2,4'-Dibromoacetophenone and 2-bromo-2-phenylacetophenone were dissolved in dimethyl sulphoxide, which was diluted to 1% (v/v) during the assays. The buffered reaction mixtures (0·75 ml., pH 3·0), containing inhibitor (0·67 mM), were allowed to stand for 16 hr. at 4° without substrate. The concentration of the inhibitors was 0·50 mM during the incubation at 45°.

In some experiments with dithiothreitol, 2,3-dimercaptopropanol, 2-mercaptoethanol and L-cysteine, enzyme samples were pretreated with the reagents (1 mM) at 4°, in a tris–HCl buffer (0·05 M, pH 8·0) for 1 hr. before assay.

Effect of pH on acid proteinase activity. Haemoglobin solution (8%, w/v) was adjusted to pH 1·8 with N-HCl, incubated for 1 hr. at 45° and dialysed against water. The solution was diluted to 4% (w/v) haemoglobin.

Buffers, in the range pH 1·8–6·5, were prepared from a mixture of phosphoric acid, formic acid and acetic acid (each 0·60 M). Portions (50 ml.) of acid were adjusted to the required pH with 4 N-NaOH and diluted to 100 ml. Cysteine (40 mM) and iodoacetamide (4 mM) were added to buffers for some experiments.

Incubation mixtures (1·0 ml.) contained phosphate–formate–acetate buffer (0·25 ml.), denatured haemoglobin solution (0·5 ml.; 4%, w/v), and enzyme sample (0·25 ml.). Cysteine (10 mM) or iodoacetamide (1 mM) was present in some experiments. The pH values of the complete incubation mixtures were measured, and used in plotting the results. The phosphate–formate–acetate buffer mixture was used in preference to citrate because preliminary experiments had shown that it had less tendency to cause precipitation of the haemoglobin substrate below pH 5.

The effect of pH on the acid proteinase activity of lysosomes was determined with a freshly prepared lysosomal–mitochondrial fraction from rabbit liver. A motor-driven homogenizer was used to suspend the tissue (28 g.) in 0·25 M-sucrose containing 0·01 M-tris–HCl, pH 7·4. The nuclear-debris fraction was removed from the homogenate by centrifugation at 600*g* for 5 min.; the lysosomal–mitochondrial pellet was then prepared by further centrifugation at 10000*g* for 20 min. The pellet was resuspended in a solution of Triton X-100 (0·1%, v/v; 280 ml.), and 0·25 ml. portions were used in the assays.

Column chromatography. Sephadex gel-filtration and ion-exchange columns were prepared as recommended by the manufacturers. They were equilibrated with the elution buffers before use, and flow rates were controlled by a peristaltic pump (Watson-Marlow Ltd.).

A column of Sephadex G-100 (2·5 cm. × 45 cm.; 220 $cm.^3$) was used to estimate molecular weight (Andrews, 1964; 1965) after calibration with substances of known molecular weight, i.e. Blue Dextran 2000, bovine serum albumin, egg albumin, α-chymotrypsinogen A, cytochrome *c* and sucrose. The buffer was 0·05 M-tris–HCl, pH 8·0, containing 0·10 M-KCl, and 1% butan-1-ol. The column was run at 4°, with a flow rate of 3·4 $cm.^3/cm.^2/hr.$

Buffers. All pH values were determined at room temperature. The pH of the tris buffers rose considerably when they were cooled to 0–4°. Buffers used in column chromatography contained 1% butan-1-ol.

Protein estimation. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin as standard. Allowance was made, when necessary, for the colour given by tris in this method.

Disk electrophoresis. A Shandon disk-electrophoresis apparatus was used by the method of Davis (1964) but without sample gels. A tris–glycine discontinuous buffer system was used, giving pH 9·5 during electrophoresis (Ornstein, 1964); the current was 5 mA/tube. The gels were cut at the position of a bromophenol blue marker zone, before staining.

Degradation of serum albumin. Crystalline bovine serum albumin (125 mg.) was incubated (16 hr. at 37°) with the purified acid proteinase (3·2 units) in a total volume of 2·5 ml., which contained sodium acetate buffer (pH 4·0, 0·2 M), L-cysteine (3 mg.) and butan-1-ol (0·1 ml.). The degraded protein (20% soluble in 2%, w/v, trichloroacetic acid) was then run on a column of Sephadex G-75 (2·0 cm. × 91 cm.; 282 $cm.^3$) in a 0·01 M-tris–HCl buffer containing 1% NaCl. Control mixtures incubated without enzyme were also run. The extinction at 254 mμ of the column effluent was plotted automatically by a Uvicord 4701 (LKB Instruments Ltd.) linked to a recorder (LKB 6520A).

RESULTS

Acid proteinase activity of rabbit liver lysosomes

The acid proteinase activity of a freshly prepared lysosomal–mitochondrial fraction of rabbit liver was determined over the range pH 2·7–6·2, in the presence of cysteine (10 mM) or iodoacetamide (1 mM). The results are presented in Fig. 1. Greatest activity was obtained at pH 3·2, and there was little difference between activities in the presence of

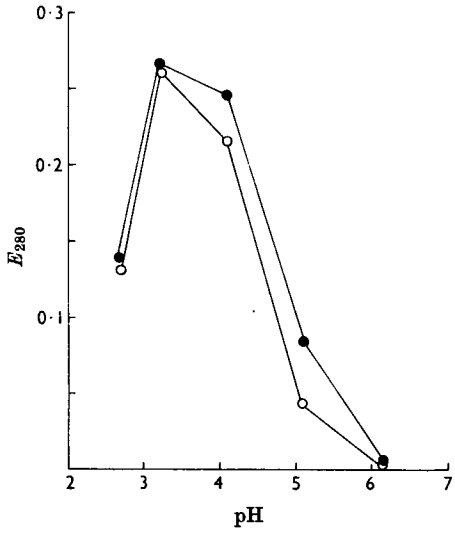

Fig. 1. Effect of pH on acid proteinase activity of a freshly prepared lysosomal–mitochondrial fraction from rabbit liver in the presence of 10mM-L-cysteine (●) or 1mM-iodoacetamide (○). See text for conditions.

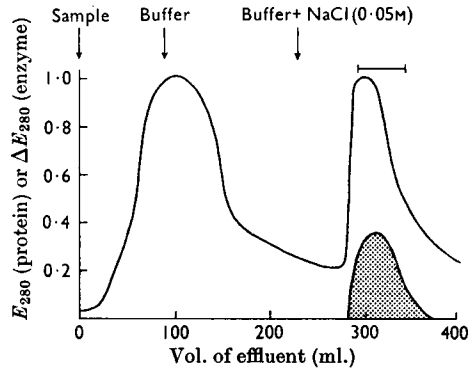

Fig. 2. Elution of protein (—) and enzyme (stippled) from a column of DEAE-Sephadex A-50 by a stepwise increase of NaCl concentration in tris–HCl buffer, *I* 0·05, pH 7·8. Samples were diluted 1:50 for the enzyme assay. The fractions marked by the bracket were pooled and retained for further purification.

L-cysteine and of iodoacetamide. The haemoglobin substrate was insoluble at, and above, pH 5.

Purification of lysosomal acid proteinase

Preparation of acetone-dried powder. Rabbits were killed by cervical dislocation. Their livers (1·7kg.) were removed immediately, mixed with frozen 0·25M-sucrose containing 0·01M-tris–hydrochloric acid buffer, pH 7·5, and passed through a mincer. The cold (0°) mince was transported to the laboratory in a Thermos flask, and all subsequent operations were carried out in a room at 4°. The mince was diluted to 20% (v/v) tissue with the buffered sucrose solution and portions (750ml.) were homogenized for 90sec., at half-speed, in an Ato-Mix blender (Measuring & Scientific Equipment Ltd.).

A nuclear-debris fraction was removed from the homogenate by centrifugation at 600*g* for 10min., after which the supernatants were centrifuged again at 10000*g* for 40min. to produce lysosomal–mitochondrial pellets. The pellets were resuspended in a small volume of water in the Ato-Mix blender, and poured into acetone (10vol.) at 4°. The mixture was stirred and allowed to stand for 1hr. before the supernatant liquid was decanted and discarded. The solid was treated twice with portions of fresh acetone and then spread on a piece of filter paper to dry, under reduced pressure, in a desiccator.

The yield of acetone-dried powder was 55g. The powder contained about 50% of the original acid proteinase activity of the homogenate, and could be stored at 4° for several months without detectable loss of activity.

Enzyme extraction. Acetone-dried powder (10g.) was suspended in a sodium formate buffer (pH 3·5, 0·2M, 200ml.) containing 20% (v/v) acetone, by use of a motor-driven homogenizer. The suspension was allowed to stand for 1hr., rehomogenized, and centrifuged at 10000*g* for 10min. The supernatants were poured into acetone (4vol.), stirred, and allowed to stand for 15min. The solid material was collected by centrifugation (10000*g*, 10min.) and allowed to drain before being resuspended in a small volume of tris–hydrochloric acid buffer (*I* 0·05, pH 7·8). The suspension was centrifuged again, the pellet washed with a little more buffer, and the combined supernatants were dialysed against the same buffer over-night.

DEAE-Sephadex. Sephadex A-50 equilibrated with the tris–hydrochloric buffer (*I* 0·05, pH 7·8) was used to form a bed 1·5cm. × 30cm. (54cm.^{3}). The dialysed enzyme extract (52ml.) was allowed to run into the ion-exchanger, which was then washed with the starting buffer. A large amount of colourless inactive protein passed unadsorbed through the column (Fig. 2). An enzymically active fraction was eluted with buffer containing 0·05M-sodium chloride, and this was freeze-dried and retained for further purification. Much of the brown material remaining on the ion-exchanger could be eluted subsequently with buffer containing 0·5M-sodium chloride.

Sephadex G-100. The Sephadex G-100 column

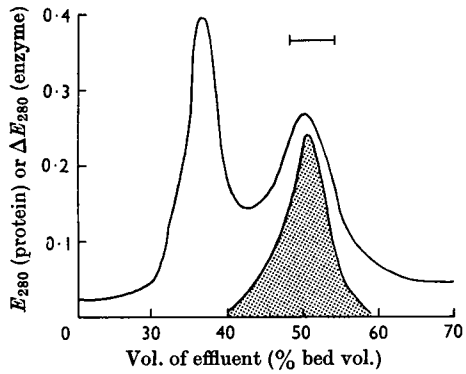

Fig. 3. Elution of protein (—) and enzyme (stippled) from a Sephadex G-100 column in tris–HCl buffer, *I* 0·05, pH 7·8. Samples were diluted 1:50 for the enzyme assay. The fractions marked by the bracket were pooled to form the final purified enzyme preparation.

Table 1. *Purification of rabbit-liver acid proteinase*

Experimental details are given in the text.

	Specific activity (units/mg. of protein)	Purification	Yield (%)
Homogenate	0·062	1	100
Acetone-dried powder	0·32	5·1	52
Concentrated extract	5·8	94	22
DEAE-Sephadex	13·2	214	8
Sephadex G-100	54	870	5

(2 cm. × 91 cm.; 282 cm.^{3}) was equilibrated with tris–hydrochloric acid buffer (*I* 0·05, pH 7·8), and the concentrated enzyme preparation, dissolved in water (7 ml.), was applied to it and eluted at a flow rate of 17 ml./hr. A plot of E_{280} and enzyme activity in the effluent (Fig. 3) showed a peak of inactive protein followed by a peak of protein with enzymic activity.

The specific activity of the enzyme in the active fraction from the Sephadex G-100 column was 860–1140 times that of the original tissue homogenate. The results of a typical experiment are summarized in Table 1.

Other purification procedures. During the development of the above scheme a number of other methods were examined, either because they are often valuable in enzyme purification or because they have been used with enzymes somewhat similar to the liver acid proteinase.

Ammonium sulphate fractionation did not produce appreciable increases in specific activity.

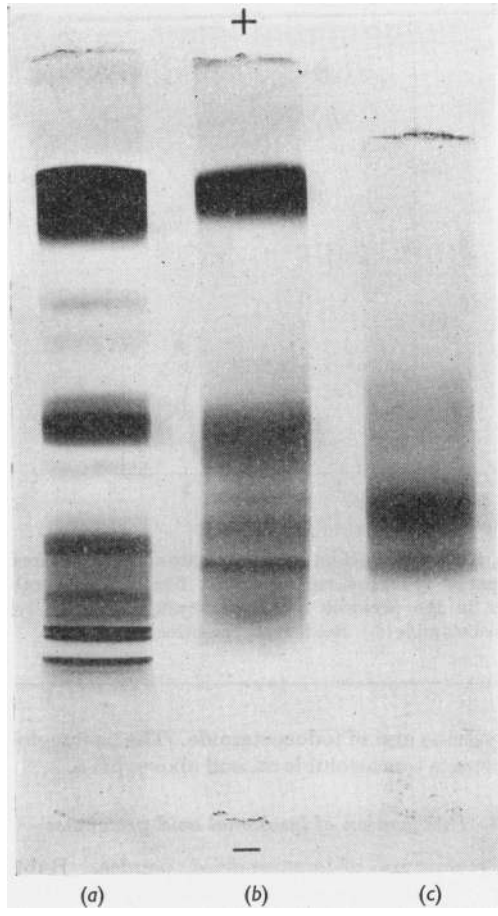

Fig. 4. Disk electrophoresis of (*a*) human serum standard, (*b*) crude enzyme preparation as applied to DEAE-Sephadex column, (*c*) purified enzyme preparation. The discontinuous tris–glycine buffer system of Davis (1964) was used, and the current was 5 mA/tube.

Columns of DEAE-cellulose gave useful purification, but were less effective than DEAE-Sephadex. The enzyme could not be eluted from this ion-exchanger with the dilute borate–phosphate buffer (21 mM, pH 8·4) used by Press, Porter & Cebra (1960).

Properties of the enzyme

Stability. Crude enzyme extracts showed little loss of activity when allowed to stand for days at 4° and pH 3–4. During the purification good recoveries of enzyme were achieved after exposure to pH 8 for a day or two, also at 4°.

The stability of the most highly purified enzyme preparations, containing 0·16 mg. of protein/ml.,

during storage in deep-freeze conditions, was improved by the presence of 20% (v/v) glycerol, and also by the use of polystyrene rather than glass containers. Under these conditions, at pH 8, there was no appreciable loss of enzymic activity during several months.

Homogeneity. Enzyme samples were run in disk electrophoresis (Fig. 4). The purified preparation contained a single major component, but it is not yet certain whether this had enzymic activity.

No acid phosphatase activity could be detected in the purified acid proteinase preparation.

Molecular weight. When the enzyme was run on the calibrated column of Sephadex G-100, the elution volume (V_e) was 101ml., intermediate between those of bovine serum albumin (89ml.) and egg albumin (110ml.). From a plot of V_e against log molecular weight the molecular weight of the enzyme was estimated at 50000–52000.

Effect of pH on activity. Under the assay conditions used, maximal enzyme activity was obtained at pH 3·2. The activity fell off sharply below this pH, but some was detectable at pH 6 (Fig. 5). The haemoglobin substrate was insoluble at, and above, pH 5.

Activators. L-Cysteine and 2-mercaptoethanol (10mM) increased the activity of some crude preparations of the enzyme by 10–20%, but did not stimulate purified preparations.

Inhibitors. A wide range of substances were tested for their ability to inhibit the acid proteinase, generally without effect (Table 2). There was no significant inhibition by *p*-chloromercuribenzoate (0·1mM), iodoacetamide (1mM), soybean trypsin inhibitor (1mg./ml.), Trasylol (2500 kallikrein inhibiting units/ml.), phenylmethanesulphonyl fluoride (1·2mg./ml.), L-1-tosylamido-2-phenylethyl chloromethyl ketone (1·2mg./ml.), ascorbate (10mM), sodium bisulphite (5mM) or 6-aminohexanoic acid (0·4M).

Dialysing the enzyme against EDTA (10mM) in tris–hydrochloric acid buffer (0·05M, pH 8·0) did not reduce its activity, nor was it inactivated by incubation with 2,4′-dibromoacetophenone (0·67 mM) or 2-bromo-2-phenylacetophenone (0·67mM).

Some substances did show inhibitory activity under the assay conditions, and others were able to inactivate the enzyme during a pretreatment at pH 8 (Table 2). In the former category were α-oxo acids such as 3-phenylpyruvic acid; the second group included dithiothreitol and some other reducing agents. Acid proteinase inactivated by exposure to 1mM-dithiothreitol was not reactivated by treatment with an excess of potassium ferricyanide or by dialysis against a large volume of tris–hydrochloric acid buffer, pH 8·0, in contact with air. The enzyme was not inactivated by oxidized dithiothreitol.

The mechansim by which the proteinase was inhibited by 3-phenylpyruvic acid was investigated by comparing reaction velocities, in the presence or absence of inhibitor (5mM), over a range of substrate concentrations (Fig. 6). The plot of $1/v$ against $1/s$ (where $v = \Delta E_{280}$/hr., and s = mg. of haemoglobin/ml.) indicated that the inhibition was competitive (Lineweaver & Burk, 1934) above 16mg. of haemoglobin/ml. At lower substrate

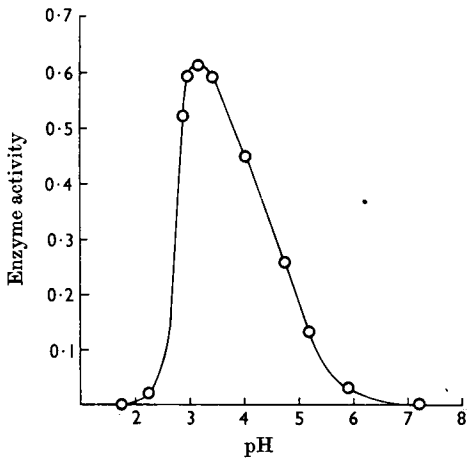

Fig. 5. Effect of pH on activity of purified lysosomal acid proteinase with haemoglobin as substrate. Values for enzyme activity above 0·3 unit were obtained by correction of measured ΔE_{280} values with a standard curve. See text for experimental details.

Table 2. *Effect of various compounds on activity of purified acid proteinase*

Results are expressed as percentage changes from the activity of control samples. Enzyme samples were pretreated with some substances at pH 8 and 4° before the assays. Experimental details are given in the text.

	Concn. (mM)	Percentage change: Without pretreatment	Percentage change: With pretreatment
2-Oxoisohexanoic acid	2	−6	—
3-Phenylpyruvic acid	4	−38	—
3-(*p*-Hydroxy)phenylpyruvic acid	4	−16	—
Indolyl-3-pyruvic acid	4	−30	—
Dithiothreitol	1	0	−80
2,3-Dimercaptopropanol	1	—	−44
2-Mercaptoethanol	1	0	−24
L-Cysteine	1	0	+4
Calcium chloride	10	−19	—
Magnesium acetate	10	−12	—
Cobalt chloride	10	−22	—

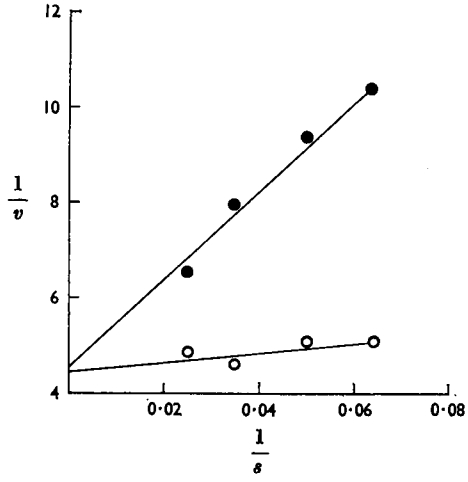

Fig. 6. Lineweaver–Burk plot showing competitive inhibition of lysosomal acid proteinase by 3-phenylpyruvic acid (5mM). The rate of enzyme action was measured at several concentrations of substrate (16, 20, 28 and 40mg./ml.) in the presence (●) or absence (○) of inhibitor. $v=\Delta E_{280}$/hr.; s=mg. of haemoglobin/ml.

concentrations (not shown in Fig. 6) the degree of inhibition decreased below that which would be predicted by extrapolation of the linear portion of the curve, giving an impression of non-competitive inhibition. Unfortunately the very low ΔE_{280} values involved could not be measured sufficiently accurately to give exact values in this part of the curve.

Substrate specificity. In addition to haemoglobin, the enzyme degraded gelatin (as photographic emulsion) and bovine serum albumin.

The lysosomal acid proteinase attacked serum albumin at only 5% of the rate at which haemoglobulin was degraded, as measured by ΔE_{280}, at pH3·2. The reaction was even slower at pH4·5. Assay mixtures containing 20mg./ml. each of haemoglobin and serum albumin gave ΔE_{280} not significantly different from those of controls without albumin, implying a very low affinity of the enzyme for albumin.

Gel-filtration of bovine serum albumin degraded by the lysosomal enzyme at pH4·0 showed a polydisperse mixture of peptides with no indication of free amino acids.

The enzyme did not hydrolyse α-*N*-benzoyl-DL-arginine-*p*-nitroaniline, at pH4·0.

Some properties of pepsin

Pepsin (twice-crystallized) had a specific activity of 265 units/mg. in the standard assay, at pH3·2.

When assays were performed with bovine serum albumin instead of haemoglobin, at the same concentration, ΔE_{280} values were not reduced.

Pepsin, in sodium phosphate buffer (pH7·0, 0·02M), was stored at 4° for 3hr. in the presence or absence of dithiothreitol (1mM). Measurements of initial and final enzymic activities showed no effect of the reducing agent on the degree of inactivation of the enzyme.

Pepsin was treated with 2,4′-dibromoacetophenone and 2-bromo-2-phenylacetophenone under the conditions used for the lysosomal enzyme. There was 80–90% inactivation by each reagent.

DISCUSSION

Proteinases can be divided into four groups: 'serine' proteinases, 'thiol' proteinases, metal-activated proteinases and acid proteinases (Hartley, 1960); it is necessary to consider how the enzyme described here can be fitted into this system. Also, it is important to decide whether this enzyme is a form of one of the previously described cathepsins.

The acid proteinase activity of the lysosomal mitochondrial fraction of rabbit liver was greatest at pH3·2 (Fig. 1). The close similarity between the pH–activity curves obtained in the presence of cysteine and of iodoacetamide shows that little of the measured activity was due to enzymes activated by cysteine and inhibited by iodoacetamide, e.g. cathepsin B and cathepsin C (EC 3.4.4.9) (Greenbaum & Fruton, 1957; Fruton, 1955).

The enzyme was eluted relatively easily from DEAE-Sephadex at pH7·8, and this may indicate that, like cathepsin D (EC 3.4.4.23) (Press *et al.* 1960), it is isoelectric at near-neutral pH. Cathepsin E, on the other hand, seems to be a very acidic protein (Lapresle & Webb, 1962) more like pepsin. The finding that the enzyme could not be eluted from DEAE-cellulose under the conditions used for cathepsin D might indicate that these are distinct proteins, but the DEAE-cellulose preparations used may have had rather different characteristics.

The molecular weight of 50000–52000 determined for the enzyme is in fair agreement with that of 58000 obtained for cathepsin D (Press *et al.* 1960). These values may be compared with that of 50000 for thyroid acid proteinase (Kress, Peanasky & Klitgaard, 1966), which is now considered to be lysosomal (Balasubramaniam & Deiss, 1965; Herveg, Beckers & De Visscher, 1966), and the approximate value of 60000 for the acid proteinase of brain (Marks & Lajtha, 1965). On the other hand, values of 35000 and 41000 obtained for acid proteinases from dental pulp (Schwabe & Kalnitsky, 1966) seem to indicate that these are distinct proteins. All of these values may be contrasted with that of 210000 for cathepsin C (Metrione, Neves & Fruton, 1966).

The pH optimum for haemoglobin breakdown was found to be 3·2. This figure is in reasonable agreement with values of 3·0 and 3·5 (Anson, 1939*b*; Press *et al.* 1960) obtained with cathepsin D of bovine spleen. Similar enzymes with pH optima in the range pH 3–4 have been reported in other tissues (Schwabe & Kalnitsky, 1966; Marks & Lajtha, 1965; Woessner, 1965*b*; Kress *et al.* 1966; Dannenberg & Smith, 1955*a*). The sharp decline in activity below pH 3 suggests that no cathepsin E is present, for this enzyme shows maximal activity at pH 2·5 (Lapresle & Webb, 1962), with serum albumin. The shape of the pH–activity curve of the pure enzyme (Fig. 5) is very similar to that obtained with the fresh unfractionated mitochondrial–lysosomal pellet (Fig. 1). This would be consistent with the possibility that nearly all of the acid proteinase activity of rabbit liver (assayed with haemoglobin) is due to the enzyme which has been isolated in this work.

The slight increase in the activity of some crude enzyme preparations in the presence of cysteine is similar to that found with cathepsin D of spleen (Anson, 1939*b*; Press *et al.* 1960), and may be due to the presence of a thiol-requiring exopeptidase.

The proteinase was not inhibited by *p*-chloromercuribenzoate, iodoacetamide or bisulphite, all of which inhibit cathepsin B (Greenbaum & Fruton, 1957; Greenbaum & Yamafuji, 1966). The enzyme was also insensitive to the polypeptide proteinase inhibitors from bovine parotid gland and soybean and this is interesting in view of reports that these substances inhibit local haemorrhagic Schwartzmann reaction (Halpern, 1964) and vascular changes in irradiated skin (Jolles & Harrison, 1966). A lysosomal enzyme may be involved in these processes, as the authors suggest, but it seems unlikely to be an acid proteinase of the type described here.

Phenylmethanesulphonyl fluoride and L-1-tosylamido-2-phenylethyl chloromethyl ketone react with some 'serine' proteinases, such as trypsin and chymotrypsin (Fahrney & Gold, 1963; Schoellmann & Shaw, 1963), but inhibition of rat brain acid proteinase by L-1-tosylamido-2-phenylethyl chloromethyl ketone has been reported (Marks & Lajtha, 1965). Neither reagent was inhibitory in the present work. It also seems improbable that the enzyme is a metal-activated proteinase since it was unaffected by EDTA and slightly inhibited by bivalent cations (like pig thyroid acid proteinase, Kress *et al.* 1966).

Since the enzyme was resistant to characteristic inhibitors of the serine, thiol and metal-activated proteinase groups it may be considered to fit well into the acid proteinase category of Hartley (1960).

Ali (1964) produced evidence that a lysosomal acid proteinase involved in the autolysis of rabbit cartilage is inhibited by 6-aminohexanoic acid, but this was without effect on the enzyme from rabbit liver.

The α-oxo acids related to leucine, phenylalanine, tyrosine and tryptophan have been shown to inhibit pepsin and chymotrypsin (Geratz, 1965). Their ability to inhibit the enzyme studied here suggests that this enzyme, too, may be specific for bonds adjacent to amino acids with aromatic or paraffinic side chains, as is cathepsin D (Press *et al.* 1960; Kress *et al.* 1966; Dannenberg & Smith, 1955*b*; Woessner, 1965*b*; Dopheide & Todd, 1964). As would be expected of a substrate analogue, the inhibition by 3-phenylpyruvic acid was competitive, at least above 16mg. of haemoglobin/ml. At lower concentrations of substrate there was a suggestion of non-competitive inhibition resembling that described for pepsin, with indolyl-3-pyruvic acid (Geratz, 1965).

The specific inactivators of pepsin (Erlanger, Vratsanos, Wassermann & Cooper, 1965; 1966), 2,4′-dibromoacetophenone and 2-bromo-2-phenylacetophenone, had no effect on the lysosomal enzyme.

Although the various thiol and dithiol reagents tested here had no inhibitory activity at pH 3 there was very striking inactivation of the enzyme when it was preincubated with dithiothreitol at pH 8 and 4°. It seems likely that this powerful reducing agent (Cleland, 1964) can break a disulphide bond essential for the maintenance of the active configuration of the enzyme, Other, less powerful, thiol reagents were also inactivators, but the fully oxidized form of dithiothreitol did not inactivate the enzyme. The role of dithiothreitol as an inactivator, here, contrasts with its use in the stabilization of many other enzymes. Pepsin was not inactivated by dithiothreitol at pH 7.

The substrate specificity of the enzyme included gelatin and serum albumin, in addition to haemoglobin. However, the rate of degradation of albumin was much less than that of haemoglobin, as was also found for spleen cathepsin D by Press *et al.* (1960), but the results presented here differ from theirs in that breakdown of albumin was not faster at pH 4·5 than at pH 3·2. The low affinity of the lysosomal enzyme for albumin was not shown by pepsin, which gave almost identical ΔE_{280} values with the two substrates, under the same conditions. Elution diagrams obtained when degraded albumin was run on a column of Sephadex G-75 were compared with those published by Lapresle & Webb (1960); they resembled that obtained with cathepsin D more closely than that for cathepsin E.

The failure of the enzyme to hydrolyse α-*N*-benzoyl-DL-arginine-*p*-nitroanilide, a chromogenic substrate of trypsin (Erlanger, Kokowsky & Cohen, 1961), further distinguishes this enzyme from cathepsin B.

The proteinase described here can be identified as a form of cathepsin D, in spite of the absence of evidence on the specificity of attack on insulin, on the grounds of its molecular weight, approximate isoelectric point, pH optimum, low affinity for serum albumin as compared with haemoglobin, and insensitivity to various possible activators and inhibitors. This conclusion supports the views of de Duve *et al.* (1962) and Woessner (1965*c*), who maintain that the 'cathepsin' characteristically found in lysosomes, and capable of degrading haemoglobin at about pH 3·5, is cathepsin D. It is hoped that the new information on the properties of cathepsin D, particularly its sensitivity to inhibition by 3-phenylpyruvic acid and to inactivation by dithiothreitol, may facilitate its identification in other biological situations and lead to a clearer knowledge of its physiological functions.

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