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ROLE OF INDIVIDUAL CATHEPSINS IN LYSOSOMAL PROTEIN DIGESTION AS TESTED BY SPECIFIC INHIBITORS

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SUMMARY

We have investigated the effect of some inhibitors on the breakdown of proteins at 38 °C and pH 5 by highly purified Triton WR-1339-filled lysosomes obtained from rat liver.

When cathepsin D (EC 3.4.23.5) was completely inhibited by pepstatin, the rate of degradation of native serum albumin was not and that of carboxymethylated serum albumin only slightly influenced. Leupeptin or antipain, used at a concentration which resulted in more than 95% inhibition of cathepsin B1 (EC 3.4.22.1) activity, strongly decreased the rate of degradation of native albumin, but affected the degradation of carboxymethylated albumin or hemoglobin only to a slight degree. Leupeptin or antipain in combination with pepstatin inhibited proteolysis of the three proteins strongly, but not completely. Omission of Cl^-, essential for cathepsin C (EC 3.4.14.1) activity, from incubation mixtures containing pepstatin, leupeptin and antipain had only a slight additional inhibitory effect. Addition of both pepstatin and monoiodoacetate fully suppressed the degradation of these protein substrates.

Some of the experiments have also been done using Triton WR-1339-filled lysosomes obtained from purified hepatocytes.

Our results indicate that (1) cathepsin D is not essential for degradation of proteins by liver lysosomal enzymes; (2) cathepsin B1 (or possibly another leupeptin-inhibited thiol protease) is the most important enzyme in the degradation of native albumin; (3) thiol enzymes, other than cathepsin B1, C or D, are involved in lysosomal protein degradation; (4) hepatocyte lysosomes contain a set of proteases sufficient for rapid and extensive degradation of albumin.

INTRODUCTION

Several studies on hemoglobin digestion by partially purified cathepsin preparations have suggested a synergistic action of cathepsins A, B and C on one hand and cathepsin D on the other hand [1–3]. From these studies, Tappel [4] postulated that during the degradation of denatured proteins by lysosomal extracts, the first bonds were split by cathepsin D, while the large peptide fragments formed were broken down by other proteases and peptidases. Earlier, Coffey and de Duve [5] had sug-

gested an important role of cathepsin D in hemoglobin breakdown, and Dingle [6] had shown its function in cartilage degradation. We have recently demonstrated the importance of thiol enzymes in the degradation of some native proteins, viz. serum albumin, cytochrome *c*, and ribonuclease [7], while Burleigh et al. [8] showed that purified cathepsin B1 could degrade native collagen. There is also strong evidence that thiol enzymes can inactivate other native enzymes such as aldolase [9], glucokinase [9], pyruvate kinase [9], tyrosine aminotransferase [10], alanine aminotransferase [11], arginase [11] and glyceraldehyde 3-phosphate dehydrogenase [12]. In this study we have examined the role of individual cathepsins in the breakdown of native and denatured serum albumin, and hemoglobin, using specific enzyme inhibitors isolated from *Streptomyces* and *Actinomyces* [13]. Pepstatin, isovaleryl-L-alanyl-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-methyl-heptanoic acid [14], inhibits in a pseudo-irreversible way cathepsins D and E [15–17]. Leupeptin, a mixture of acetyl- and propionyl-L-leucyl-L-leucyl-L-argininal [18], and antipain, *S*-1-carboxy-2-phenylethyl-carbamoyl-L-arginyl-L-valyl-L-argininal [19], inhibit cathepsin B, probably a mixture of cathepsins B1 and B2 [20]. Both substances are competitive inhibitors of many proteases [18, 21].

Our experiments were done at pH 5. As discussed in our previous report [7], this pH is within the range where maximal proteolysis of several protein substrates occurs. Another reason for the use of pH 5 was that albumin has a compact conformation at this pH, similar to that of the native protein (see ref. 7).

Most experiments on degradation of proteins by an extract of liver lysosomes by others and by ourselves have been done with enzymes derived from a mixture of different cell types. Besides hepatocytes, which form 60% of the cells and more than 90% of the mass of the tissue, liver contains some other cell types, which are often loosely called Kupffer cells [22]. Kupffer cells contain a relatively large amount of some lysosomal enzymes like arylsulphatase [23], acid DNAase [24], and cathepsin D, as found in our laboratory (J. Wester and C. H. C. M. Buys, unpublished). Therefore, we have also investigated the degradative capacity of highly purified lysosomes derived from purified hepatocytes.

MATERIALS AND METHODS

Preparation of lysosomal extracts

a. Whole liver. Triton WR-1339-filled lysosomes were prepared by a modification of the method of Trouet [25], as described by Kussendrager et al. [26]. The lysosomal fractions were purified about 50-fold with respect to the homogenate as determined by their specific activities of acid phosphatase. Fractions were frozen in liquid nitrogen and stored at −20 °C for periods up to 1 month. Before use in the incubation mixtures, they were diluted with an equal volume of deionized water, and the lysosomal membranes were disrupted by 3 cycles of freezing in liquid nitrogen and thawing at 38 °C.

b. Hepatocytes. Hepatocytes were isolated from rats, treated with Triton WR-1339, according to the method of Hommes et al. [27]. In this method lysozyme is used to loosen the cells and the use of proteolytic enzymes is avoided. The purity of the preparation was checked by phase-contrast microscopy. The cells were disrupted by 15–25 strokes in a Potter–Elvehjem homogenizer, and the Triton WR-1339-filled

lysosomes were isolated as described for whole liver. The specific activity of acid phosphatase did not differ significantly between both types of lysosomal preparations.

Determination of protein breakdown

All incubations were done at 38 °C in stoppered tubes. Usually the incubation mixture contained an amount of lysosomal material equivalent to 0.9 units of acid phosphatase activity (approx. 0.45 mg lysosomal protein), 5.0 mg protein substrate, 39 μmoles acetate buffer (pH 5.0), in a volume of 3.1 ml. Dithiothreitol was used at a concentration of 2 mM, monoiodoacetate at 10 mM, $AgNO_3$ at 0.5 mM, pepstatin at $4.8 \cdot 10^{-6}$ M, leupeptin at $3.3 \cdot 10^{-5}$ M and antipain at $2.2 \cdot 10^{-5}$ M. Pepstatin was added dissolved in 10 μl of methanol. The lysosomal preparation, native albumin and hemoglobin were fully soluble under these conditions, but carboxymethylated albumin was used as a suspension.

The degree of enzymatic hydrolysis was calculated from the increase in ninhydrin value, corrected for blanks containing enzyme or substrate alone. Substrate blanks were always small if not negligible, and their ninhydrin values did not increase during incubation. The degree of splitting is expressed as a percentage of the ninhydrin values obtained after complete acid hydrolysis. The procedure is described more fully in our previous report [7].

In some cases the enzymatic hydrolysis of native and denatured albumin was also followed by electrophoresis on a 7.5% polyacrylamide gel in the presence of sodium dodecylsulphate according to Weber et al. [28]. With this method no breakdown of substrate was detectable in the absence of enzyme even after 24 h incubation.

Preparation of carboxymethylated albumin

1 g bovine serum albumin was incubated at 38 °C under nitrogen in 100 ml of a solution containing 8 M urea, 0.1 M Tris (pH 8.9), 25 mM EDTA and 25 mM dithiothreitol. After 3 h, 1 g of recrystallized monoiodoacetic acid was added and the solution was stirred at 20 °C in the dark during 45 min. The carboxymethylated albumin was dialyzed extensively against 0.5 mM acetate buffer (pH 5.4). The protein content was determined according to Lowry et al. [29].

Determination of enzymes

Acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) was determined according to Gianetto and de Duve [30] with some minor modifications. Cathepsin B1 (EC 3.4.22.1) was determined according to Method I given by Barrett [31] with some minor modifications. Cathepsin C (dipeptidyl peptide hydrolase, EC 3.4.14.1) was determined according to Bouma and Gruber [32]. Cathepsin D (EC 3.4.23.5) was determined according to the $A_{280\ nm}$ method, as described by Barrett [31].

Materials

Bovine serum albumin was obtained from Poviet Producten NV, Amsterdam, the Netherlands; bovine hemoglobin was prepared according to Anson [33]. Dithiothreitol was obtained from Calbiochem, San Diego, U.S.A.; monoiodoacetic acid from E. Merck, Darmstadt, Germany; Triton WR-1339 from Sigma, St. Louis, Mo., U.S.A. Pepstatin, leupeptin and antipain were generous gifts from Dr H. Umezawa, Microbial Chemistry Research Foundation, Tokyo, Japan.

RESULTS

Effect of pepstatin, leupeptin and antipain on the activity of some cathepsins

We assayed the inhibitory effect of pepstatin on cathepsin D in order to determine the concentration necessary for our experiments on protein degradation. As Fig. 1A shows, $4.8 \cdot 10^{-6}$ M (10 μg per incubation) completely inhibits cathepsin D activity. Since this is also a large molar excess in relation to the enzyme, this concentration was used in our experiments on protein breakdown. At this concentration,

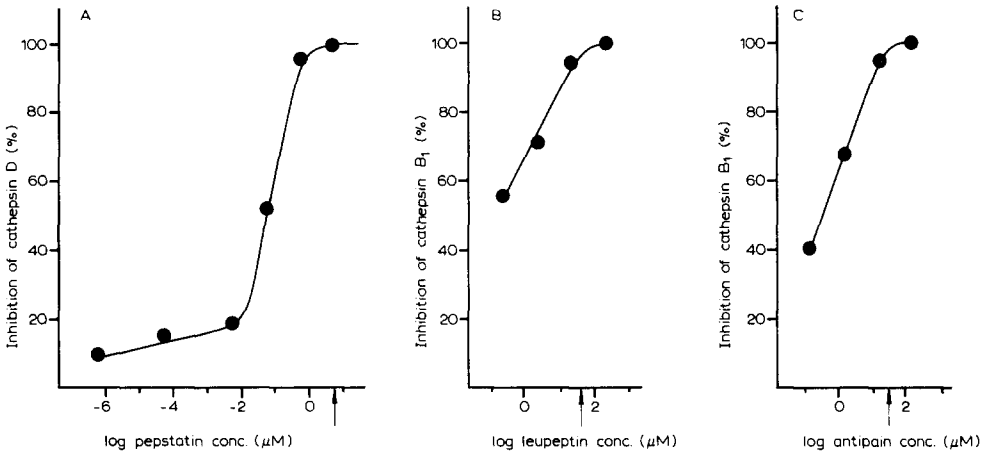

Fig. 1. Effect of pepstatin, leupeptin and antipain on the activities of cathepsins B1 and D. Enzyme activities were determined as described under Materials and Methods. The values are the averages of two independent determinations. (A) inhibition of cathepsin D activity by pepstatin. (B) Inhibition of cathepsin B1 activity by leupeptin. (C) Inhibition of cathepsin B1 activity by antipain. The arrows indicate the concentrations used during incubation of protein with lysosomal extracts.

pepstatin had no effect on cathepsins B1 or C. The effects of leupeptin and antipain on cathepsin B1 are given in Fig. 1B and 1C, respectively. We confirmed the inhibition by leupeptin and antipain of cathepsin B, a mixture of B1 and B2 (substrate: benzoyl-arginine amide), which had been shown before by Ikezawa et al. [20]. In our experiments on the degradation of proteins by lysosomal extracts, we used a concentration of $3.3 \cdot 10^{-5}$ M (50 μg per incubation) leupeptin and $2.2 \cdot 10^{-5}$ M (50 μg per incubation) antipain; this resulted in more than 95% inhibition of cathepsin B1, when measured against Bz-DL-Arg-Nan (substrate concentration 2.4 mM), while cathepsins C and D were not affected. Used at these concentrations, leupeptin and antipain gave about 60% inhibition of the hydrolysis of benzoyl-arginine amide (substrate concentration 50 mM). More than 90% inhibition was obtained at inhibitor concentrations of about 1 mM, which suggests that cathepsin B2 also can be completely inhibited by leupeptin and antipain if the inhibitor concentration is sufficiently high in relation to that of the substrate.

Since these cathepsin B inhibitors act competitively on many proteases, the degree of inhibition might depend upon the nature and the concentration of the substrates used. We have, therefore, determined the concentrations of leupeptin and

antipain necessary for maximal inhibition of the breakdown of denatured serum albumin by lysosomal extracts, which contained sufficient pepstatin to block cathepsin D activity completely. In this system, maximal inhibition of proteolytic activity was obtained when $1.0 \cdot 10^{-5}$ M leupeptin or $6.5 \cdot 10^{-6}$ M antipain were added.

Effect of inhibitors on lysosomal proteolysis

All experiments, except for those where thiol groups were blocked, were done in the presence of 2 mM dithiothreitol. This addition is necessary to standardize conditions, since, as we showed earlier [7], hydrolysis in the absence of added thiols largely depends on the history of the lysosomal preparation. Fig. 2 shows the degradation of native serum albumin. The presence of pepstatin had hardly any effect on

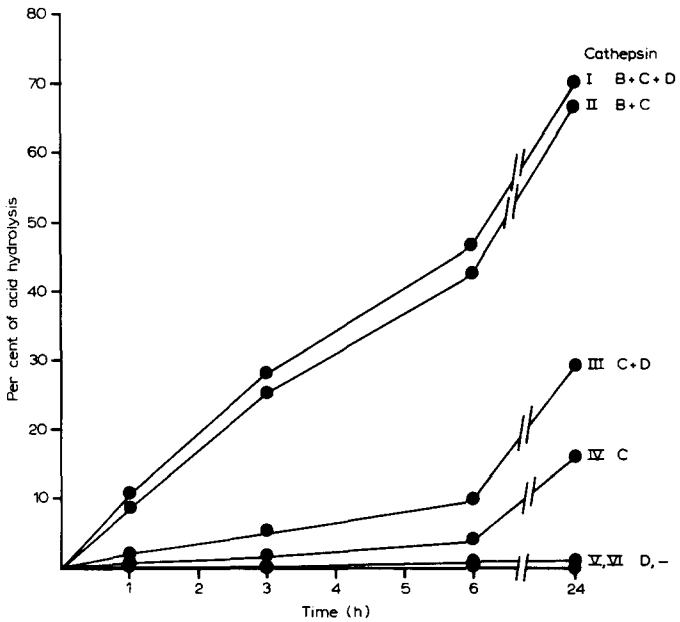

Fig. 2. Degradation of native bovine serum albumin by lysosomal extracts at pH 5.0. Incubation conditions and assays are described in Materials and Methods. The values are the average of three independent determinations. The ninhydrin value obtained by acid hydrolysis was 8.8 μmoles leucine equivalents per mg dry protein. I, no inhibitor present; II, 4.8 μM pepstatin; III, 33 μM leupeptin, 22 μM antipain, or both; IV, 4.8 μM pepstatin, and 33 μM leupeptin or 22 μM antipain or both B1 inhibitors; V, 10 mM monoiodoacetate; VI, 10 mM monoiodoacetate and 4.8 μM pepstatin. The cathepsins, shown at the right hand of the figure, indicate enzymes known to be active under the incubation conditions; here B stands for B1 and B2.

the rate of degradation of this substrate, ruling out an important contribution of cathepsin D. The cathepsin B1 inhibitors leupeptin and antipain on the other hand, caused a strong reduction in the rate of degradation. Combination of pepstatin and monoiodoacetate prevented the hydrolysis of native albumin completely, whereas in the presence of pepstatin together with leupeptin or antipain some activity was retained. This difference is probably due to some other thiol protease(s) than cathepsin B1. The possibility that the remaining activity was due to incomplete inhibition

of cathepsin B1, could be ruled out, since a 10-fold increase (to $3.3 \cdot 10^{-4}$ M) of the leupeptin concentration had little effect. The complete absence of degradation of native albumin in the presence of monoiodoacetate is striking. Cathepsin D, which is not inhibited by monoiodoacetate, is apparently not able to split this protein in its compact, native conformation to acid-soluble products.

Fig. 3 shows the degradation of denatured (carboxymethylated) serum albumin. The degradation of carboxymethylated albumin proceeds faster than that of the native protein. Addition of pepstatin slowed down the rate of degradation consider-

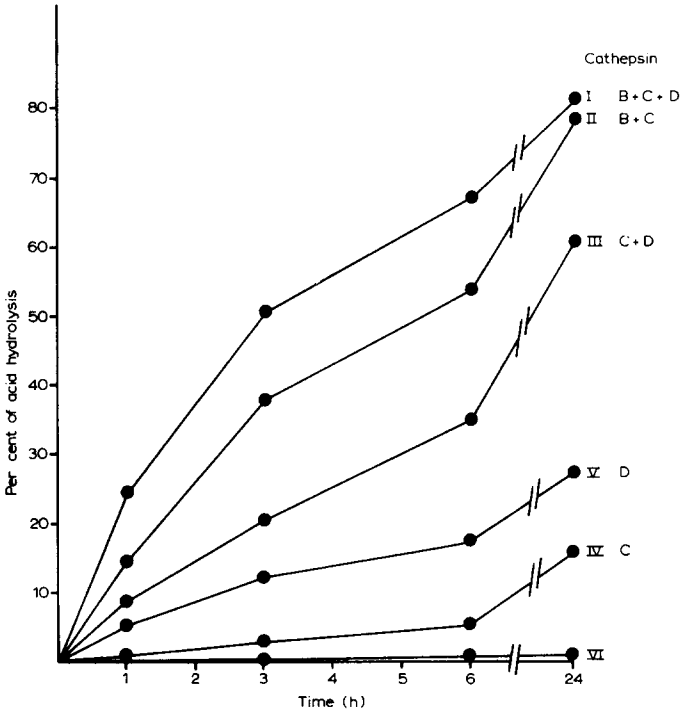

Fig. 3. Degradation of carboxymethylated bovine serum albumin by lysosomal extracts at pH 5.0. The values are the average of three independent determinations. The ninhydrin value obtained by acid hydrolysis was 8.8 μmoles leucine equivalents per mg dry protein. For details and further explanations see legend to Fig. 2.

ably, but had no effect on the ultimate extent of hydrolysis. A comparable reduction in the rate of degradation was obtained by adding the cathepsin B1 inhibitors. The effect of these inhibitors on the breakdown of denatured albumin is much smaller than in the experiments on the native protein. As expected, cathepsin D could degrade the denatured protein. Together, monoiodoacetate and pepstatin prevented protein degradation completely, whereas in the presence of both pepstatin and leupeptin or antipain some hydrolysis still occurred. Comparison of Curves IV and V with III (Fig. 3) clearly shows a synergistic effect of cathepsin D and those enzymes, which were not inhibited by leupeptin or antipain. (The same holds for native serum albumin as can be seen in Fig. 2).

Fig. 4 shows the degradation of bovine hemoglobin. The results are similar to those obtained with carboxymethylated serum albumin. The inhibitory effect of both pepstatin and leupeptin or antipain (Curve IV in Fig. 4) on the degradation of this protein is, however, smaller. This finding indicates an important role of some thiol enzymes other than cathepsin B1 or a lack of inhibition of this enzyme by leupeptin

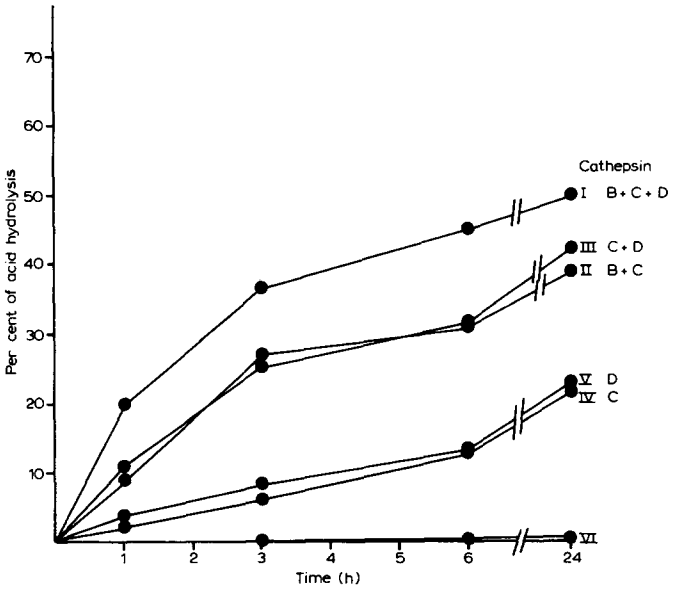

Fig. 4. Degradation of bovine hemoglobin by lysosomal extracts at pH 5.0. The values are the average of two independent determinations. The ninhydrin value, obtained by acid hydrolysis was 9.9 μmoles leucine equivalents per mg dry protein. For details and further explanations see legend to Fig. 2.

or antipain at the concentrations used. In order to clarify this point and to identify a possible role of cathepsin C, we have done experiments described in Fig. 5. A 10-fold increase in leupeptin concentration hardly reduced protein degradation. Omission of Cl^-, essential for cathepsin C activity [34], from the medium also had little effect. Like monoiodoacetate, Ag^+ completely prevented protein degradation. Therefore, the slow but significant degradation of proteins, occurring when cathepsin B1, B2, C and D are blocked, must probably be ascribed to some other thiol-dependent lysosomal enzymes.

In order to visualize possible high-molecular weight intermediates, we analyzed the reaction products at different times by polyacrylamide–sodium dodecylsulfate electrophoresis. The results, some of which are shown in Fig. 6, confirmed and extended those obtained by determining acid-soluble ninhydrin-positive material. Comparison of Gel A with Gel B shows that cathepsin D alone does not attack native serum albumin, even after an incubation time of 24 h. Comparison of Gel A and Gel C suggests that some of the thiol proteases, active in the presence of both pepstatin and leupeptin, are endopeptidases.

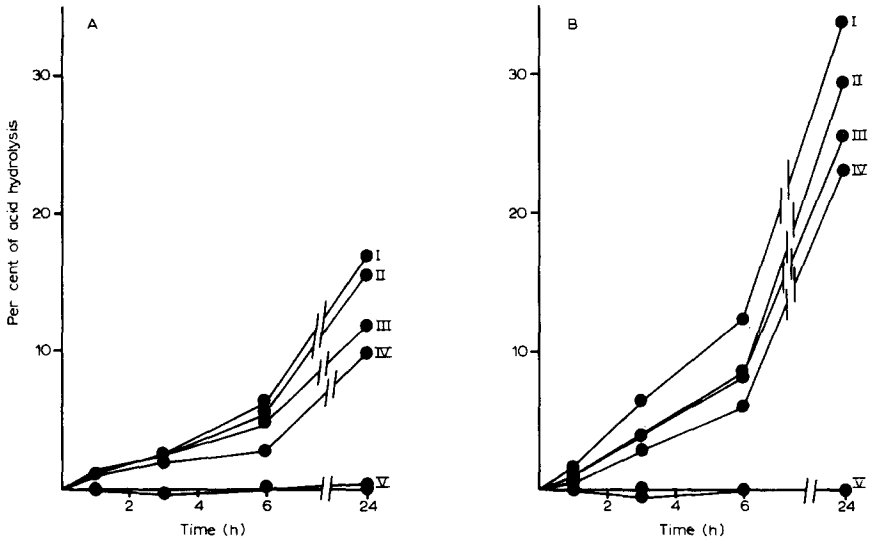

Fig. 5. Degradation of carboxymethylated albumin (A) and hemoglobin (B) by lysosomal extracts at pH 5.0. The incubation conditions and assays are described in Materials and Methods. The values are the average of three independent determinations. I, 4.8 μM pepstatin, 33 μM leupeptin, 10 mM NaCl; II, 4.8 μM pepstatin, 33 μM leupeptin, no NaCl added; III, 4.8 μM pepstatin, 330 μM leupeptin, 10 mM NaCl; 1V, 4.8 μM pepstatin, 330 μM leupeptin, no NaCl added; V, 4.8 μM pepstatin and 0.5 mM $AgNO_3$.

Stability of some lysosomal enzymes

Studies on the extent of protein degradation after prolonged incubation, can be done only if the cathepsins retain their activities. We have studied the stability of cathepsins B1, C and D under our incubation conditions. The results are shown in Table I. In accordance with the results of Coffey and de Duve [5], we observed that

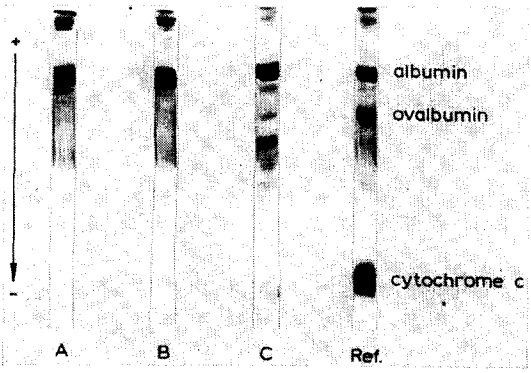

Fig. 6. Polyacrylamide gels obtained after electrophoresis in the presence of sodium dodecylsulphate of samples taken from incubation mixtures containing native albumin and lysosomal extracts. The incubation conditions and the method of electrophoresis are described under Materials and Methods. In order to detect low concentrations of intermediates the gels have been overloaded. (A) Sample taken at $t = 0$. (B) Sample taken after 24 h incubation in the presence of 10 mM monoiodoacetate (Curve V of Fig. 2). (C) Sample taken after 24 h incubation in the presence of 4.8 μM pepstatin and 33 μM leupeptin (Curve IV of Fig. 2). Ref: references albumin, ovalbumin and cytochrome *c*.

TABLE I

STABILITY OF LYSOSOMAL ENZYMES DURING INCUBATION AT pH 5.0

Lysosomal enzymes were incubated without substrates under the conditions described in Materials and Methods. At the times indicated samples were frozen in liquid nitrogen. After thawing, enzyme activities were determined as described under Materials and Methods. The values are the averages of at least two independent determinations. Values are expressed as the percentages of the activities present at the beginning of the incubation.

Incubation conditions	Enzyme Incubation time (h)	Percentage of initial activity							
		Acid phosphatase		Cathepsin B1		Cathepsin C		Cathepsin D	
		4	24	4	24	4	24	4	24
Dithiothreitol		95	91	101	83	94	89	100	95
Monoiodoacetate		94	64	—	—	—	—	92	94
Dithiothreitol + pepstatin		97	81	88	65	91	104	—	—
Dithiothreitol + leupeptin		98	92	—	—	64	59	89	84
Dithiothreitol + antipain		101	90	—	—	45	42	99	97

cathepsin D was quite stable, even in the absence of substrate. The same holds for the other proteases, cathepsin B1 and C, and for acid phosphatase. Evidently, these enzymes, in their native conformation, do not contain peptide bonds, which are accessible to the lysosomal cathepsins. Cathepsin C is partially inactivated in the presence of leupeptin or antipain. This inactivation was observed only if the enzyme had been preincubated with the inhibitors as shown in the table. Without preincubation, no inhibition was observed, even if the leupeptin or antipain concentration was raised 6-fold.

Degradation of native albumin by lysosomal enzymes obtained from purified hepatocytes

Extracts of Triton WR-1339-filled lysosomes from purified hepatocytes were isolated as described under Materials and Methods. The specific activity of acid phosphatase was nearly the same in lysosomes isolated from whole liver and from purified hepatocytes. The specific activity of cathepsin D in hepatocyte lysosomes was about 60% of that of lysosomes from whole liver, indicating that the cathepsin D concentration in hepatocytes is much lower than that in Kupffer cells. This is in accordance with unpublished results of Wester and Buys in our laboratory. The pattern of degradation of native albumin by hepatocyte lysosomal extracts was identical to that shown in Fig. 2 for whole liver. This shows that hepatocyte lysosomes contain a set of proteases sufficient for extensive and rapid hydrolysis of proteins.

DISCUSSION

Our results on the inhibition of cathepsin D by pepstatin are in agreement with several other publications [15–17], which have shown that pepstatin is an ideal cathepsin D inhibitor. Cathepsin D has an extremely high affinity for pepstatin, making the inhibition practically irreversible. Pepstatin had, in the concentration used,

no effect on cathepsin B1 or C. The inhibition by antipain of cathepsin B1 (as tested with Bz-DL-Arg–Nan as substrate) has not been reported before, although Ikezawa et al. [15, 20] reported that antipain and leupeptin inhibited the hydrolysis of benzoyl-arginine amide, a substrate of both cathepsins B1 and B2. Barrett [35] has shown that leupeptin inhibits human cathepsin B1. Leupeptin and antipain inhibit some other proteases, and thus probably also cathepsin B1, in a competitive way; under our incubation conditions their inhibition was, however, practically complete. These inhibitors do not influence cathepsins C or D, but a prolonged incubation caused a partial inactivation of cathepsin C. This unexplained phenomenon will influence our analysis to a minor degree only, since at least 40% of the enzyme remains active. According to Ikezawa et al. [20] antipain, in contrast to leupeptin, also inhibits cathepsin A. It is not known whether pepstatin, leupeptin or antipain have any effect on lysosomal enzymes besides those tested.

Pepstatin at a concentration which inhibited cathepsin D completely, did not influence the breakdown of native albumin, and had little effect on the final degree of splitting of other protein substrates. Evidently, the role of this enzyme can to a large extent be mimicked by other lysosomal enzymes, at least at pH 5.

If serum albumin is a representative example, our results show an essential all-or-none character of the breakdown of native proteins, indicating that the first splits are rate determining. In the first stages at least, cathepsin B1, or another leupeptin sensitive thiol enzyme, is essential. Our earlier results [7] on the digestion of ribonuclease also point to an all-or-none character.

Digestion of the protein substrates could be blocked completely by a mixture of pepstatin, and monoiodoacetate or Ag^+. This probably indicates that all proteases, except for cathepsin D, contain essential thiol groups.

In the presence of pepstatin, leupeptin and/or antipain the breakdown of protein substrates was very considerably retarded, but not stopped, even if Cl^- were omitted; antipain was just as effective as leupeptin, indicating a minor role of cathepsin A. This means that lysosomes contain (a) thiol enzyme(s) other than cathepsin A, B1, B2, C and D, which can degrade both native and denatured proteins. The results of the polyacrylamide gel electrophoresis make the presence of endopeptidases among these unknown enzymes likely. Possible candidates are a thiol cathepsin, resembling, but according to De Lumen and Tappel not identical to, cathepsin B1 [36]; and a thiol cathepsin L, reported to have a high specific activity in the hydrolysis of protein substrates [37]. Moreover, thiol-dependent carboxypeptidases [38, 39], and a thiol-dependent aminopeptidase (unpublished results of Van der Laan, Bouma and Gruber) may contribute to protein breakdown. It is unlikely that the action of exopeptidases alone is responsible for the formation of discrete fragments from a compact protein like albumin at pH 5.

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