Biochimica et Biophysica Acta, 422 (1976) 380—389

BBA 67699

PROTEOLYSIS OF CANINE APOLIPOPROTEIN BY ACID PROTEASES IN CANINE LIVER LYSOSOMES

TSUGUHIKO NAKAI, PATRICIA S OTTO and THOMAS F WHAYNE, Jr

Cardiovascular Research Program, Oklahoma Medical Research Foundation and the Departments of Medicine and Biochemistry and Molecular Biology, University of Oklahoma College of Medicine, Oklahoma City, Okla 73104 (U S A)

(Received July 16th, 1975)

Summary

Canine liver lysosomes were purified by sucrose discontinuous density gradient centrifugation and then ruptured by sonication to obtain the soluble fraction This soluble lysosomal fraction, which contained a 25-fold increase in acid phosphatase activity per mg of total protein when compared with the original homogenate, was incubated with a subfraction ($1\ 110 < d < 1\ 210$ g/cm^3, HDL_3) of canine high density lipoproteins (HDL) at pH 3 8 HDL_3 proteolysis by lysosomal proteases, measured as the release of peptides and amino acids by the ninhydrin reaction, followed hyperbolic curves with straight lines (r = 0 99) obtained on Lineweaver-Burk plots K_m calculated from the Lineweaver-Burk plot was 635 μg of HDL_3 protein per 0 5 ml of incubation mixture Optimum HDL_3 proteolysis was observed from pH 3 8 to 4 5 Incubation with the other subcellular organelle fractions did not result in HDL_3 proteolysis To evaluate the effects of enzyme inhibitors, iodoacetate, *p*-chloromercuribenzoate (both specific for the endopeptidase, cathepsin B (EC 3 4 22 1)) and pepstatin (specific for the endopeptidase, cathepsin D (EC 3 4 23 5)) were tested Iodoacetate and *p*-chloromercuribenzoate inhibited HDL_3 proteolysis 100% and bovine serum albumin proteolysis 65% Pepstatin inhibited HDL_3 proteolysis 45% and bovine serum albumin proteolysis 70% The in vitro data presented support the hypothesis that hepatic lysosomes play an important role in HDL_3 catabolism in the dog Furthermore, results obtained from enzyme inhibition studies suggest that a specific lysosomal endopeptidase, cathepsin B, may play the key role in HDL_3 proteolysis

Introduction

The definite subcellular site(s) and the mechanisms involved in apolipoprotein catabolism have not been established, although recent studies have

suggested that the major catabolic site is the lysosome Rachmilewitz et al. [1] and Stein et al. [2] studied the subcellular localization of rat ^{125}I-labeled high density lipoproteins (HDL) and ^{125}I-labeled very low density lipoproteins (VLDL) in rat liver by electron microscopic radioautography Silver grains were identified over various cytoplasmic organelles but marked grain density was found only over dense bodies, many of which could be classified as secondary lysosomes

We have previously investigated the catabolic rate and fate of apolipoprotein (Apo) A-I, one of the three major plasma apolipoproteins [3] Canine Apo A-I was chosen because lipoprotein family A (mainly present in high density lipoprotein) is the major carrier of cholesterol in the dog [4,5], thereby providing a large animal model for testing this metabolism. Preliminary studies in our laboratory have suggested that the liver and the lysosomes in liver parenchymal cells are important organ and subcellular catabolic sites, respectively, of canine Apo A-I [3] The latter was studied by direct subcellular organelle isolation and demonstration of labeled apolipoprotein uptake in dogs. At one day after injection of ^{125}I-labeled Apo A-I, total radioactivity in the liver was 4 8 times greater than in the kidney and 20 0 times greater than in the spleen In the subcellular fractions of liver, the radioactivity was predominantly recovered in the lysosomal fraction

The purpose of the present report was to investigate in vitro, the hydrolysis of apolipoproteins by lysosomal proteolytic enzymes (e g cathepsins) Because of solubility problems of canine Apo A-I or Apo HDL_3 (the starting material for Apo A-I purification) in the assay system pH of 3 8, native canine HDL_3 (1 110 $< d <$ 1 210) was used as substrate

Methods

Isolation of HDL_3 After a 16-h fast, blood was obtained from healthy male mongrel dogs weighing approx. 20 kg and fed a commercial dog food which provides approx 26% of calories as protein, 22% as fat and 52% as carbohydrate (Purina Dog Chow). The blood was placed in centrifuge tubes containing EDTA, 1.0 mg/ml of blood. One part of 0 25 M EDTA and 0 5 M phosphate buffer (Na_2HPO_4, KH_2PO_4, pH 7 5) was added to 49 parts of plasma to give in plasma an EDTA concentration of 0 005 M and a phosphate buffer concentration of 0 01 M To inhibit bacterial growth, polymyxin B sulfate was added to give a serum concentration of 25 units/ml [6] The plasma density was adjusted to d = 1 110 g/cm^3 with solid KBr and the plasma was then centrifuged in a Type Ti 60 rotor on the L or L2 preparative ultracentrifuge (Beckman Instruments Inc , Palo Alto, California) for 22 h at 105 000 × g After 22 h, the top 4 ml was removed by the tube-slicing technique HDL_3 was then isolated by adjusting the density of the infranatant solution with solid KBr to d = 1 210 g/cm^3 and centrifuging at 105 000 × g for 22 h The top 4 ml containing HDL_3 was removed by tube slicing and the HDL_3 was washed by repeat ultracentrifugation at least three times at d = 1 210 g/cm^3 to remove contaminating serum albumin, followed by dialysis against double distilled water at 4°C Studies with HDL_3 as substrate were performed immediately following its isolation

Preparation of soluble lysosomal fraction After a 16-h fast, healthy male mongrel dogs (about 20 kg) were anesthetized with sodium thiopental (Abbott Laboratories, North Chicago, Ill). Following perfusion through the portal vein with 0 25 M sucrose/1.0 mM EDTA solution (sucrose/EDTA solution, pH 7 2, 4°C), the fresh liver tissue was chopped into pieces about 1 cm^3 in size About 120 g chopped liver tissue was mixed with 1 2 l of sucrose/EDTA solution The mixture was homogenized in a blender (Waring Products, New Hartford, Connecticut) for 30 s at high speed. Homogenates were filtered through eight layers of coarse cheesecloth into a chilled container All procedures were performed at 4°C The canine liver homogenate was centrifuged according to the proce-

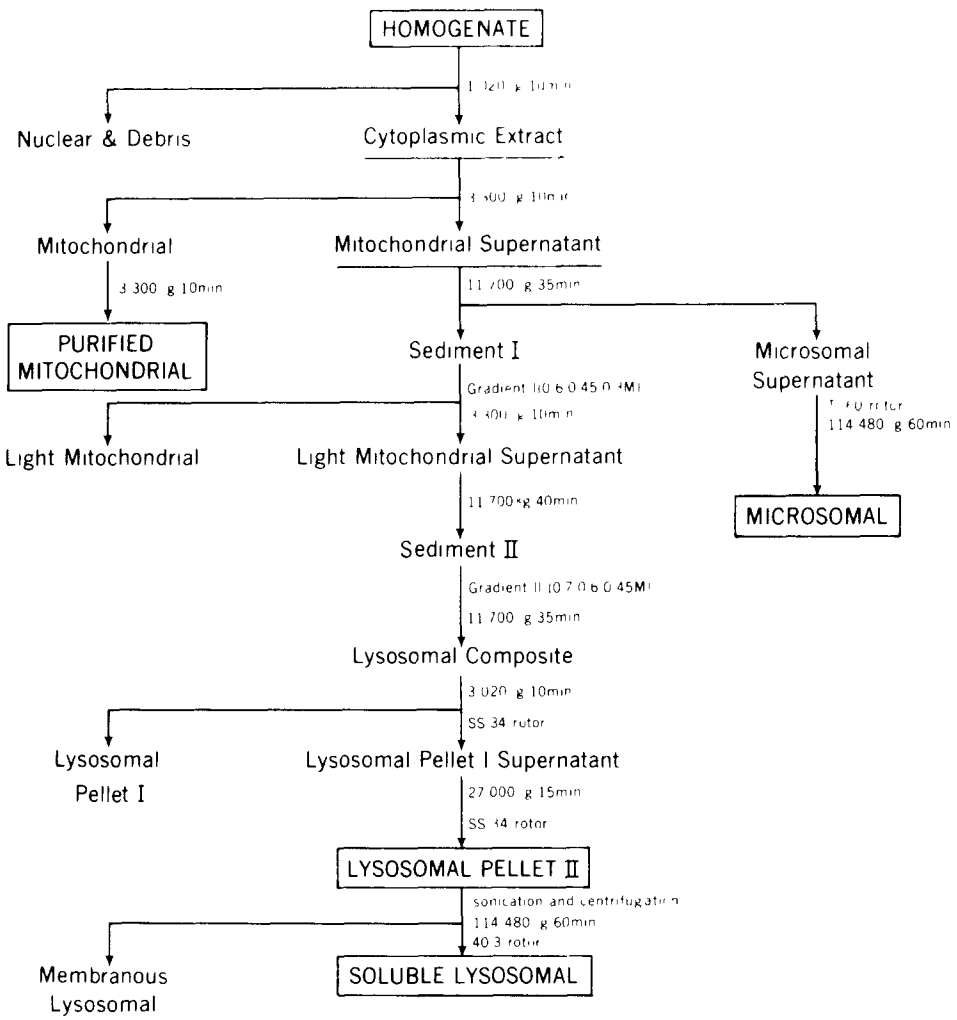

Fig 1 Centrifugation scheme for subcellular organelle preparation is shown the gradients were prepared in three discrete layers and were as follows from bottom to top Gradient I 70 ml of 0 60 M sucrose, 60 ml of 0 45 M sucrose and 50 ml of 0 30 M sucrose in which a portion of Sediment I was suspended Gradient II 70 ml of 0 70 M sucrose, 60 ml of 0 60 M sucrose and 50 ml of 0 45 M sucrose in which a portion of Sediment II was suspended

dure of Ragab et al [7] (Fig 1), using a discontinuous sucrose gradient The Sorvall RC-2 centrifuge was used with the GSA rotors for all centrifugations except for centrifugations of lysosomal composite and lysosomal pellet I supernatant The final lysosomal pellet was suspended in 20 ml of sucrose/EDTA solution and sonicated with a Biosonic Macroprobe (Bronwill Scientific, Rochester, N Y), intensity 50, for 30 s at 4°C The sonicated lysosomal fraction was centrifuged in a Type 40 3 rotor on the Beckman L or L2 preparative ultracentrifuge for 60 min at 114 480 × *g* to remove the insoluble material The supernatant of the sonicated lysosomal fraction (soluble lysosomal fraction) was used for incubation with HDL_3 Each fraction obtained during the isolation procedure was characterized by measuring the enzyme activities of succinate dehydrogenase according to the method of Pennington [8], acid phosphatase by the method of Berthet and De Duve [9] and glucose-6-phosphatase by the method of Hubscher and West [10] Inorganic phosphate released in acid phosphatase and glucose-6-phosphatase assays was determined according to Fiske and SubbaRow [11] Protein content was measured by the method of Lowry et al [12], using canine serum albumin (Fraction V, Mann Research Laboratories, Inc., New York, N Y) as standard

Basic proteolysis assay system The system for assay of proteolytic activity in the soluble lysosomal fraction consisted of the following 0 25 ml of the soluble lysosomal fraction (containing 50—60 μg of total protein and an acid phosphatase activity of 10 nmol phosphate released per 1.0 ml soluble lysosomal fraction per min at 37°C) and 0 25 ml of HDL_3 (containing 1.0 mg of protein in 0 1 M sodium acetate buffer, pH 3 8) or bovine serum albumin (crystallized and lyophilized, Sigma Chemical Co., St Louis, Mo.) containing 0 375 μmol protein in 0.1 M sodium acetate buffer, pH 3 8, as substrate Incubations were carried out for 60 min at 37°C and pH 3.8 The reaction was stopped by addition of 1 0 ml of 10% trichloroacetic acid. To diminish adsorption of trichloroacetic acid-soluble products on the material precipitated by trichloroacetic acid, the samples were kept overnight at 4°C [13] After centrifugation, 1 0 ml of the supernatant was used for the ninhydrin reaction according to Rosen [14], leucine was used as standard for estimation of reactive amino groups. All assays were determined in duplicate The ninhydrin values obtained were corrected for blanks containing enzyme source (soluble lysosomal fraction) and substrate alone Substrate blanks showed no change over the incubation period, enzyme blanks did show change which was corrected for by subtraction Proteolytic enzyme activity was expressed as μmol amino group equivalents released per 60 min incubation in the basic proteolysis assay system

Assay of proteolytic activity in homogenates, and mitochondrial and microsomal fractions with HDL_3 as substrate Proteolytic activity was also assayed using liver homogenate, mitochondrial fraction and microsomal fraction as enzyme sources, with HDL_3 as substrate, following sonication of these subcellular fractions The basic proteolysis assay system was used except that enzyme concentrations were increased so that measurable activities could be obtained for all fractions Enzyme concentrations, expressed as μg of protein per 0 25 ml were as follows homogenate, 205, mitochondrial fraction, 198, soluble lysosomal fraction, 190, and microsomal fraction, 218

Effect of pH on HDL_3 and bovine serum albumin proteolysis by the soluble lysosomal fraction Different pH values of 5 0, 4 5, 4 2, 4 0 and 3 8 were obtained using 0 1 M sodium acetate, adjusted by adding glacial acetic acid The pH 7 0 and 6.0 solutions were Michaelis' barbital/sodium acetate buffer The pH 3 0, 2 0 and 1 0 solutions were 0 1 M sodium acetate solution, the pH of which was adjusted by adding glacial acetic acid and HCl

Effect of enzyme modifiers on proteolytic activity in soluble lysosomal fraction with HDL_3 and bovine serum albumin as substrate The effects of iodoacetate, *p*-chloromercuribenzoate, dithioerythritol and pepstatin* were tested The soluble lysosomal fraction was preincubated with iodoacetate, *p*-chloromercuribenzoate or dithioerythritol for 15 min at 37°C following the iodoacetate inhibition system described in Fig 3. Pepstatin was added as a methanolic solution to the assay vessel After evaporation of the methanol, 0.25 ml of soluble lysosomal fraction was added and the mixture was preincubated for 30 min at 37°C HDL_3 (0 25 ml) or bovine serum albumin (0 25 ml) were then added as substrate

Metal ions and EDTA effect on proteolytic activity in soluble lysosomal fraction with HDL_3 as substrate The soluble lysosomal fraction of the basic proteolysis assay system was dialyzed against 10 mM sodium acetate buffer, pH 5 0 This enzyme preparation was used for evaluation of the effect of metal ions ($CaCl_2$, $MgCl_2$, $MnCl_2$ and $FeCl_3$) and EDTA on proteolytic activity in soluble lysosomal fraction with HDL_3 as substrate The metal ions and EDTA were in a concentration range of 1 μM to 10 mM and were preincubated with HDL_3 for 15 min

Results

Characterization of subcellular fractions of canine liver (Table I) The average specific activity of acid phosphatase, succinate dehydrogenase and glucose-6-phosphatase in the whole liver homogenate, mitochondrial fraction, soluble lysosomal fraction and microsomal fraction are shown in Table I The soluble lysosomal fraction, when compared with the whole liver homogenate, was purified 25 0 ± 11 7 (± S D)-fold with respect to specific acid phosphatase activity The protein yield, presented as mg protein per g wet weight of liver in whole liver homogenate, mitochondrial fraction, soluble lysosomal fraction and microsomal fraction was 212 0 ± 26 0, 9 0 ± 4 9, 0 13 ± 0 05 and 0 74 ± 0 18, respectively.

Proteolytic activity against HDL_3 in subcellular fractions Apo HDL_3 and Apo A-I (purified by column chromatography of totally delipidated Apo HDL_3 on Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N J.)) were insoluble in any of the following solutions at pH 3 8—4 5 0 1 M sodium acetate buffer, 0 05 M citrate buffer and Michaelis' barbital/sodium acetate buffer [15] and were not hydrolyzed effectively by acid proteases in canine liver lysosomes Therefore intact HDL_3 was chosen as substrate, which was completely soluble in 0 1 M sodium acetate buffer (pH 3 8) Using HDL_3 as sub-

* Kindly supplied by the Institute of Microbial Chemistry, Tokyo, Japan, through Dr Jordan Tang, Laboratory of Protein Studies, Oklahoma Medical Research Foundation

TABLE I

SEPARATE CHARACTERIZATIONS OF SUBCELLULAR FRACTIONS OF CANINE LIVER FROM EACH OF FIVE SEPARATE DOGS

Marker enzyme assays were used to assess purification of subcellular fractions as follows Acid phosphatase for lysosomes, succinate dehydrogenase for mitochondria and glucose-6-phosphatase for microsomes

Subcellular fraction	Specific activity ($\times 10^3 \pm$ S D)		
	Acid phosphatase*	Succinate dehydrogenase**	Glucose-6-phosphatase***
Homogenate	1 ± 1	14 ± 8	30 ± 11
Mitochondrial	4 ± 3	56 ± 39	11 ± 7
Soluble lysosomal	26 ± 4	0	2 ± 2
Microsomal	1 ± 2	5 ± 6	76 ± 41

* Expressed as μmol phosphate liberated per mg subcellular fraction protein per min
** Expressed as μmol formazan formed per mg subcellular fraction protein per min
*** Expressed as μmol phosphate liberated per mg protein per min

strate, the soluble lysosomal fraction had a proteolytic activity of 1 75 μmol amino group equivalents released per mg subcellular fraction protein per h. This proteolytic activity was greater by a factor of 23 times than the proteolytic activity of the homogenate, while the mitochondrial fraction and the microsomal fraction did not show any significant activity

Time course of HDL_3 and bovine serum albumin proteolysis by soluble lysosomal fraction Linear kinetics for HDL_3 and bovine serum albumin proteolysis by the soluble lysosomal fraction were achieved up to 2 h (r = 0 99), an incubation time of 60 min was used in the investigations reported

Effect of substrate concentration on HDL_3 and bovine serum albumin proteolysis by soluble lysosomal fraction Proteolysis of HDL_3 and bovine serum albumin by lysosomal acid proteases conformed to a hyperbolic curve (Fig 2) The apparent K_m calculated from a Lineweaver-Burk plot of the hyperbolic curve was 635 μg of HDL_3 protein per 0 5 ml of incubation mixture (46 μM based on Apo A-I molecular weight of 28 000) and 374 μM in bovine serum albumin proteolysis

Effect of pH on HDL_3 and bovine serum albumin proteolysis by soluble lysosomal fraction Under our assay conditions, the optimal rate of HDL_3 proteolysis was observed at pH 4 2 and no activity was found at pH 7 0, 6 0, 2 0 and 1 0. Proteolysis of bovine serum albumin was optimal at pH 3 5

Effect of inhibitors on HDL_3 and bovine serum albumin proteolysis by soluble lysosomal fraction Iodoacetate (Fig 3) and *p*-chloromercuribenzoate (both specific for cathepsin B (EC 3 4 22 1)) inhibited HDL_3 proteolysis 100% at concentrations greater than 50 μM, while bovine serum albumin proteolysis was inhibited 68% by iodoacetate (Fig 3) and 63% by *p*-chloromercuribenzoate, (each at a concentration of 100 μM) The maximum inhibition of HDL_3 and bovine serum albumin proteolysis by pepstatin (specific for cathepsin D (EC 3 4 23.5)) was approx. 45 and 70%, respectively, at a concentration higher than 10 μM Dithioerythritol (1 mM) activated HDL_3 proteolysis approx 2-fold Control proteolytic activity in the basic proteolysis assay system was 0 157 μmol amino group equivalents released with HDL_3 as substrate and

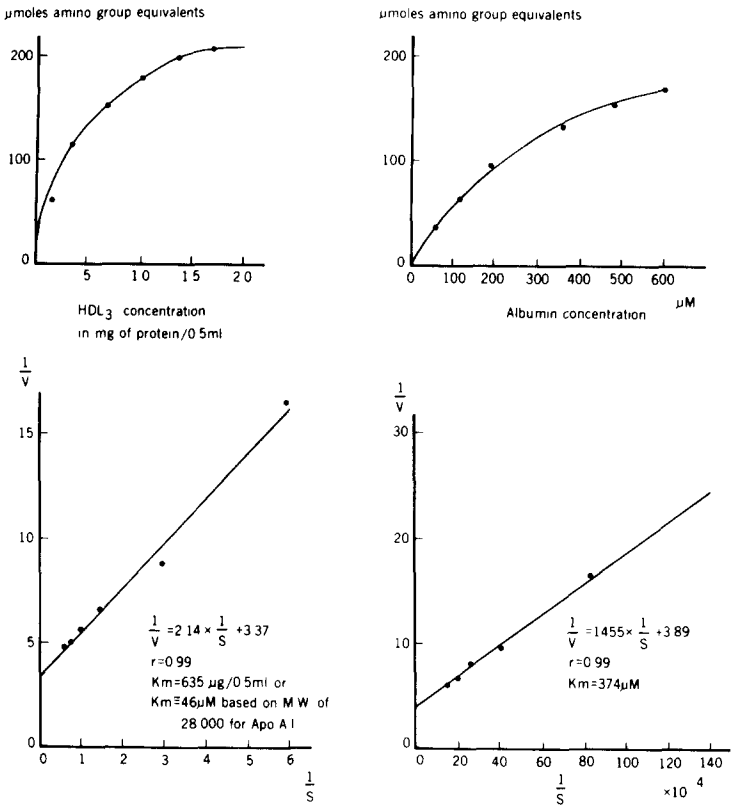

Fig 2 The effect of substrate concentration on HDL_3 and bovine serum albumin proteolysis by the soluble fraction from one canine liver the basic proteolysis assay system was modified by the use of increasing substrate concentrations Estimation of K_m values made by Lineweaver-Burk plots also is shown

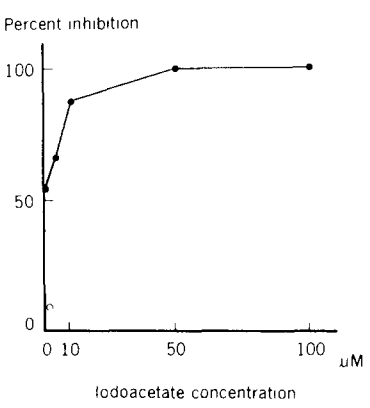

Fig 3 Effect of iodoacetate on HDL_3 (solid line) and bovine serum albumin (broken line) proteolysis by the soluble lysosomal fraction from one canine liver the assay system contained 0 25 ml of soluble lysosomal fraction, 0 25 ml of either HDL_3 or bovine serum albumin solution as substrate and 0 5 ml of iodoacetate solution with increments in iodoacetate concentration The protein content of the soluble lysosomal fraction, HDL_3 and bovine serum albumin was the same specified for the basic proteolysis assay system The soluble lysosomal fraction was preincubated with iodoacetate for 15 min at 37°C HDL_3 or bovine serum albumin were then added and the mixture was incubated for 60 min, after which 0 5 ml of 20% trichloroacetic acid was added to terminate proteolysis

0.185 μmol amino group equivalents released with bovine serum albumin as substrate

Effect of metal ions and EDTA on proteolytic activity in soluble lysosomal fraction from one canine liver with HDL_3 as substrate Among the metal ions tested, $MnCl_2$ inhibited proteolysis 25.9% and $FeCl_3$ inhibited proteolysis 70 0%, at concentrations greater than 1 mM With EDTA, $CaCl_2$ and $MgCl_2$, HDL_3 proteolysis showed approx 120% of control activity at concentrations of greater than 0 01, 10 and 0 01 mM, respectively. Control proteolytic activity with HDL_3 as substrate was 0 108 μmol amino group equivalents released in the basic proteolysis assay system

Discussion

The soluble fraction of our purified canine liver lysosomes had a specific activity of acid phosphatase approx 25 times greater than the original homogenate Even though electron microscopic studies of purified lysosomal pellets indicated the presence of mitochondria (the numerical ratio of mitochondria/lysosomes was (0 7—1.0)/1 0), the sonicated mitochondrial and microsomal fractions exhibited very minimal proteolytic activity for HDL_3, when compared with the soluble lysosomal fraction

Cathepsins are a major group of proteolytic enzymes in the soluble fraction of lysosomes and consist of exopeptidases and endopeptidases. The pH optima of exopeptidases (cathepsin A (EC not yet assigned), and C (EC 3 4 14 1)) are greater than 5 0 [16,17] while those of the major lysosomal endopeptidases (cathepsins B and D) are in the range 3 0—4 5 with hemoglobin as substrate [18—20] Cathepsin E (EC 3 4 23 5), a minor lysosomal endopeptidase, has a very low pH optimum (around 2 5) and a limited tissue distribution (polymorphonuclear leukocytes, bone marrow and spleen) [19], suggesting that the physiologic role of this endopeptidase may be more limited than cathepsins B and D In the degradation of proteins, endopeptidases first hydrolyze the protein to peptides, which are then degraded to amino acids by exopeptidases [17,21] Although the pH optimum of HDL_3 proteolysis by our soluble lysosomal fraction ranged from 3.8 to 4 5, a pH value of 3 8 was selected in order to evaluate mainly proteolysis by endopeptidases and to minimize the effect of exopeptidases Under the conditions used in our incubations, proteolysis of HDL_3 and bovine serum albumin followed Michaelis-Menten kinetics, suggesting formation of enzyme-substrate complexes [22] during the course of proteolysis

Different cathepsins in the soluble fraction of purified lysosomes may participate in the proteolysis of HDL_3 as suggested by effects of enzyme modifiers such as metal ions, iodoacetate, *p*-chloromercuribenzoate, pepstatin and dithioerythritol Misaka and Tappel [23] reported the inhibitory effect of $MnCl_2$ on cathepsin B and $FeCl_3$ on cathepsins B and D In our experiments, $MnCl_2$ and $FeCl_3$ inhibited HDL_3 proteolysis, indicating that both cathepsin B and D are participating in HDL_3 proteolysis Iodoacetate and *p*-chloromercuribenzoate probably inhibit cathepsin B by acting on a thiol group in the enzyme active site [20,24,25] Iodoacetate and *p*-chloromercuribenzoate (both specific for cathepsin B) [20,24] completely inhibited HDL_3 proteolysis, while bovine

serum albumin proteolysis was inhibited 65% Pepstatin obtained from cultured broth of actinomycetes is a specific inhibitor of acid proteases and has the structure isovaleryl-L-valyl-L-valyl-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-heptanoic acid [26] Pepstatin does not inhibit a variety of neutral proteases but exhibits a strong inhibitory effect on pepsin [27] and cathepsin D [28—30]. In our study, pepstatin inhibited HDL_3 proteolysis 45% and bovine serum albumin proteolysis 70%. Even though little is known about the specificities of cathepsins B and D [21,24], the responses of HDL_3 and bovine serum albumin (which was chosen as representative of a heterologous protein) proteolysis to enzyme modifiers suggest that the susceptibilities of these proteins to cathepsin B and/or D differ Furthermore, our observations suggest that the endopeptidase, cathepsin B, plays a more significant role in HDL_3 proteolysis, while the endopeptidase, cathepsin D, plays a more significant role in bovine serum albumin proteolysis The role of cathepsin B for HDL_3 proteolysis is supported also by the activation of this reaction by dithioerythritol, which is reported to activate cathepsin B [13,31] by thiol group activation

In conclusion, the in vitro data presented support our hypothesis that hepatic lysosomes play an important role in HDL_3 catabolism in the dog Furthermore, our data suggest that a key mechanism of this catabolism is proteolysis of the HDL_3. Finally, results obtained from enzyme inhibition studies suggest that the lysosomal endopeptidase, cathepsin B, may play the major role in HDL_3 proteolysis

Acknowledgements

The authors gratefully acknowledge the suggestions and criticisms of Drs Reagan H Bradford and Jordan Tang. This work was performed during Dr Nakai's tenure as a Postdoctoral Research Fellow of the Oklahoma Heart Association and was supported in part by a U S. Public Health Service grant awarded to the Oklahoma Medical Research Foundation (5-S01-RR05538).

References

1 Rachmilewitz, D , Stein, O , Roheim, P S and Stein, Y (1972) Biochim Biophys Acta 270, 414—425
2 Stein, O , Rachmilewitz, D , Sanger, L , Eisenberg, S and Stein, Y (1974) Biochim Biophys Acta 360, 205—216
3 Nakai, T and Whayne, T F (1975) Clin Res 23, 326A
4 Solyom, A , Bradford, R H and Furman, R H (1971) Biochim Biophys Acta 229, 471—483
5 Solyom, A , Bradford, R H and Furman, R H (1971) Biochim Biophys Acta 236, 563—570
6 Whayne, T F and Felts, J M (1970) Circ Res 27, 941—951
7 Ragab, H , Beck, C , Dillard, C and Tappel, A L (1967) Biochim Biophys Acta 148, 501—505
8 Pennington, R J (1961) Biochem J 80, 649—654
9 Berthet, J and De Duve, C (1951) Biochem J 50, 174—181
10 Hubscher, G and West, G R (1965) Nature 205, 799—800
11 Fiske, C H and SubbaRow, Y (1925) J Biol Chem 66, 375—400
12 Lowry, O H , Rosebrough, N J , Farr, A L and Randall, R J (1951) J Biol Chem 193, 265—275
13 Huisman W , Bouma, J M W and Gruber, M (1973) Biochim Biophys Acta 297, 98—109
14 Rosen, H (1957) Arch Biochem Biophys 67, 10—15
15 Michaelis, L (1931) Biochem Z 234, 139—141
16 Taylor, S L and Tappel, A L (1974) Biochim Biophys Acta 341, 112—119

17 Goettlich-Rieman, W , Young, J O and Tappel, A L (1971) Biochim Biophys Acta 243, 137—146
18 Barrett, A J (1970) Biochem J 117, 601—607
19 Lapresle, C (1971) Tissue Proteinases (Barrett, A J and Dingle, J T , eds), p 135, North-Holland Publishing Co , Amsterdam
20 Otto, K (1971) Tissue Proteinases (Barrett, A J and Dingle, J T , eds), p 1, North-Holland Publishing Co , Amsterdam
21 Huang, F L and Tappel, A L (1971) Biochim Biophys Acta 236, 739—748
22 White, A (1973) Principles of Biochemistry, p 225, McGraw-Hill, New York
23 Misaka, E and Tappel, A L (1971) Comp Biochem Physiol 38B, 651—662
24 Huisman, W , Lanting, L , Doddema, H J , Bouma, J M W and Gruber, M (1974) Biochim Biophys Acta 370, 297—307
25 Snellman, O (1971) Tissue Proteinases (Barrett, A J and Dingle, J T , eds), p 29, North-Holland Publishing Co , Amsterdam
26 Morishima, H , Takita, T , Aoyagi, T , Takeuchi, T and Umezawa, H (1970) J Antibiot 23, 263—265
27 Umezawa, H , Aoyagi, T , Morishima, H , Matsuzaki, M , Hamada, M and Takeuchi, T (1970) J Antibiot 23, 259—262
28 Ikezawa, H , Aoyagi, T , Takeuchi, T and Umezawa, H (1971) J Antibiot 24, 488—489
29 Woessner, J F (1972) Biochem Biophys Res Commun 47, 965—970
30 Smith, R and Turk, V (1974) Eur J Biochem 48, 245—254
31 Cleland, W W (1964) Biochemistry 3, 480—482