Neutral Proteases and Cathepsin D in Human Articular Cartilage

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ABSTRACT Proteolytic enzymes have been studied in extracts of human articular cartilage by the use of micromethods. The digestion of hemoglobin at pH 3.2 and of cartilage proteoglycan at pH 5 was shown to be due chiefly to cathepsin D. Cathepsin D was purified 900-fold from human patellar cartilage. Its identity was established by its specific cleavage of the B chain of insulin. At least six multiple forms of cathepsin D are present in cartilage; these corresponded to bovine forms 4–9. Cathepsin D had no action on proteins at pH 7.4. However, cartilage extracts digested proteoglycan, casein, and histone at this pH. The proteolytic activities against these three substrates were purified about 170-, 160-, and 70-fold, respectively. Each activity appeared in multiple forms on DEAE-Sephadex chromatography. The three activities appear to be different since cysteine inhibited casein digestion, aurothiomalate inhibited histone digestion, and neither inhibited proteoglycan digestion. Tests with a wide range of inhibitors and activators suggest that these three activities differ from other neutral proteases described in the literature.

INTRODUCTION

The primary events which trigger the destruction of the cartilage matrix in joint disease are the subject of numerous hypotheses (1–3). However, it is generally thought that the damage is ultimately produced by lysosomal hydrolases. One of the main problems under investigation in our laboratory is which hydrolases are responsible. Human synovial tissue and fluid contain cathepsin D, neutral protease (4), collagenase (5, 6), and hyaluronidase (7); but little is known about these enzymes in human cartilage. No hyaluronidase has been detected in human cartilage, even in osteoarthritic lesions (8), and only vague evidence has been presented for the presence of collagenase (9). There is some evidence for neutral protease activity in human cartilage (10, 11), but cathepsin D is the only protease that has been clearly demonstrated (12, 13).

In recent years considerable experimental evidence has led to the consideration of the acid cathepsin D as the chief agent in the degradation of cartilage matrix (14–16), although the pH of the matrix is close to neutrality (12). It was assumed that cathepsin D could act in the supposedly acidic environment surrounding the chondrocytes (2) or that the enzyme could act at neutral pH, since its higher pH optimum on proteoglycan than on hemoglobin might extend its activity into the neutral pH range (17).

In a recent study (12) we demonstrated that a cathepsin D-type enzyme was present in two to three times the normal amount in primary osteoarthritic human cartilage. This enzyme was the predominant protease in the articular cartilage extracts acting on proteoglycan and hemoglobin at acid pH. These human cartilage extracts also degraded proteoglycan extensively at neutral pH. This neutral activity appeared at the tail end of the cathepsin D pH-activity curve and was therefore attributed to the residual activity of cathepsin D at neutral pH. This attribution was in keeping with the results of other workers in the field. In particular, Dingle et al. (14, 17) showed that antiserum to cathepsin D was capable of inhibiting the autolytic degradation of cartilage at pH 7.1. These studies of cathepsin D in cartilage have been summarized elsewhere (18).

Recently, it was discovered that the action of cathepsin D did not extend into the neutral pH range and that the activity seen on proteoglycan was due to a contaminating neutral protease (18, 19). This result was based on improved purification of cathepsin D from bovine uterus. This result suggested a review of the situation in cartilage, paying particular attention to the

Received for publication 11 October 1973 and in revised form 28 November 1973.

presence of neutral protease(s) which might be involved in the breakdown of proteoglycan. In the process cathepsin D was further purified and positive identification was made on the basis of substrate specificity.

METHODS

Enzyme preparations. Patellae were removed at autopsy from 40 male patients, age 45–70 yr, with no recognizable joint disease. The patellae were kept on ice, trimmed, washed with ice-cold saline solution, and frozen until used. Extracts for enzymatic studies were prepared from the articular cartilage of these patellae as previously described (12). The final extracts in 5 mM phosphate buffer, pH 8.0, were stored in the cold with added penicillin and streptomycin (10,000 U/ml). Cathepsin D (forms 4, 5, and 6 combined) was prepared from bovine uterus as described in reference 20.

Protease assays. The microassay for hemoglobin digestion and the definition of a cathepsin unit have been described (12). Proteoglycan subunit (PGS) [1] was prepared from bovine nasal cartilage by the method of Hascall and Sajdera (21). The digestion of this substrate was followed by viscometry and sedimentation (12, 13). Caseinolytic activity was assayed using azocasein substrate (Schwarz/Mann, Orangeburg, N. Y.). Azocasein was first boiled in 0.2 M citrate buffer, or 0.2 M phosphate buffer of the desired final pH. The final incubation mixture contained 50 μl azocasein (1–4% w/v), 25 μl 0.2 M buffer, and 25 μl enzyme extract. After incubation at 37°C for 2 or 20 h, 100 μl 0.5 M trichloroacetic acid (TCA) was added. The pellet formed upon incubating this mixture for 15 min, 20°C, was removed using a model 152 Microfuge (Beckman Instruments, Inc., Palo Alto, Calif.). Supernate (100 μl) and 200 μl 0.5 M NaOH were mixed in a 0.4 ml cuvette. The azo dye was measured by its absorbance at 450 nm. Then 60 μl Folin-Ciocalteau reagent (1:3 in water) was added and tyrosine content was determined at the end of 10 min by the absorbancy at 660 nm. Blanks were prepared with enzyme and substrate incubated separately and combined after the TCA addition. The digestion of calf thymus histone was according to the method of Davies, Krakauer, and Weissmann (22). The total incubation volume was reduced to 0.2 ml and incubation was for 2 h, 37°C. After incubation, 0.2 ml sulfosalicylic hydroquinol reagent was added to measure arginine at 500 nm; or 0.1 ml 10% TCA for the measurement of tyrosine.

Products of PGS digestion. PGS (3.75 mg/ml) was digested under varying conditions in a total incubation volume of 0.2 ml. The digestion products were fractionated by adding 50 μl 7.5% cetyl pyridinium chloride, and 10 min later, 75 μl 10% TCA. Pellets were removed by "Microfuge" sedimentation of the chilled suspension. The pellets were dissolved in 2 M KCl, reprecipitated, and washed with ETOH. Uronic acid content of the pellet and supernatant was determined by the method of Bitter and Muir (23). Sedimentation coefficients of the PGS and the soluble and precipitable forms were determined by the method of Pita and Mueller (24). The samples were diluted to 0.04 mg/ml to minimize concentration effects on the value of S (25). Fragments of molecular weight below 6,000 daltons were isolated by dialysis through VisKing tubing in a 1 ml dialysis cell (Chemical Rubber Co., Cleveland, Ohio).

[1] *Abbreviations used in this paper:* EPP, 1,2-epoxy-3-phenoxypropane; PGS, proteoglycan subunit.

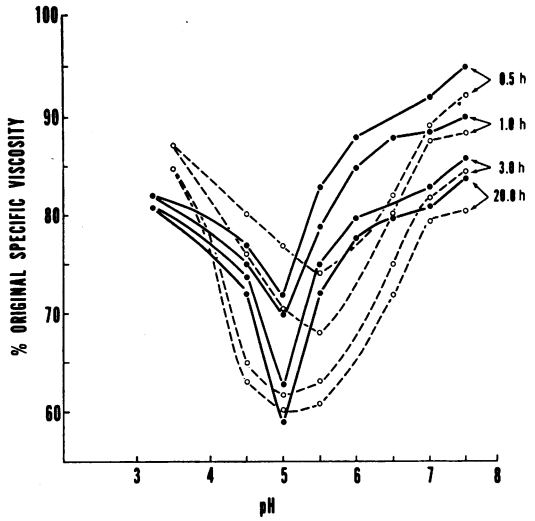

FIGURE 1 pH profile of PGS degradation as measured by microviscosimetry. PGS having a sedimentation coefficient of 15S was used as substrate. ○, patellar extract containing 0.07 U cathepsin D; ●, purified bovine cathepsin D, 0.07 U.

Inhibitor studies. All inhibitors, except 1,2-epoxy-3-phenoxy-propane (EPP) (Eastman Kodak Co., Rochester, N. Y.), were preincubated at room temperature with the enzyme extracts for 15 min before adding the substrate. For blanks, enzyme and inhibitor were incubated together and then combined with substrate after TCA addition. Pepstatin, a generous gift from Dr. S. Itakura (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan) was dissolved in ETOH (1 mg/ml) and 1 μl of this solution was added to 200 μl incubation mixture.

EPP was diluted 1:5 with ETOH and 5 μl were added to 200 μl incubation mixture. ETOH was added to blanks in equal amount. Enzyme-inhibitor incubations continued for 1 h at 37°C before substrate was added for the final assay of protease activities.

Purification of cartilage enzyme extracts. Cartilage extracts were lyophilized, and the powder was dissolved in 10 ml 5 mM phosphate buffer, pH 7, and dialyzed against this buffer. The sample was applied to a 0.9 × 35-cm Sephadex G-100 column which had been equilibrated with the same buffer. The column was eluted with this buffer, and 1-ml portions were collected and assayed for the enzyme activities. The active fractions were combined and lyophilized. The dry powder was dissolved in, and dialyzed against, 5 mM sodium phosphate buffer, pH 7. Chromatography on a 0.9 × 35-cm column of DEAE-Sephadex A25 followed the procedure used earlier for the separation of multiple forms of cathepsin D (20) except that the pH was lowered from 8.8 to 7.0. Elution was performed in steps of increasing phosphate buffer strength of 10, 20, 30, 40, and 50 mM at pH 7. The final purification step involved disc electrophoresis (26, 27) of the active DEAE peaks. Polyacrylamide-gel columns of 7 cm length and 7.5% gel concentration were used. A set of four gels was run in parallel; one gel was stained with Coomassie blue (28), and the other three were sliced into 30 slices. Corresponding slices were combined and eluted with 5 mM phosphate buffer, pH 7.

Protein content was estimated by absorbancy at 280/260

TABLE I

Effect of Pepstatin on the Digestion of PGS

	pH 5				pH 7	
	Patellar extract		Bovine cathepsin D		Patellar extract	
	Control	Pepstatin	Control	Pepstatin	Control	Pepstatin
5 min	94*	99	89	100	88	90
10 min	89	98	84	99	80	84
30 min	78	94	79	96	68	74
60 min	73	90	74	96	64	70
120 min	70	86	74	93	61	67
180 min	67	82	74	91	59	66
240 min	62	77	72	91	57	64
20 h	61	79	69	86	57	61

Pepstatin, 1 μg, was added to 0.15 ml PGS (15S) containing enzyme. Different preparations of PGS gave different limiting viscosities in the controls at 20 h. Inhibition by pepstatin resulted in a much slower loss of viscosity at pH 5, but there was only slight inhibition at pH 7.

* Results are expressed as percent of initial viscosity of PGS.

nm (29). For the acrylamide-gel extracts, blanks had to be prepared by extracting gel slices that contained no protein. Cathepsin D was characterized by its action on the B chain of insulin. Incubation, fingerprinting and identification of the peptides were carried out using the same methods used for bovine cathepsin D (20, 30).

RESULTS

Digestion of protein substrates by patellar extracts. The action of patellar enzymes on PGS is shown in Fig. 1. PGS is digested (reduction in viscosity) over a wide range of pH from 3.2 to 7.5. The optimum pH for digestion is 5.0. Purified bovine cathepsin D is included for comparison; its pH curve is similar but shows a sharper optimum at pH 5. These patterns are similar to those published earlier (12, 13). However, care was taken in the present study to adjust the enzymes so that there was equal cathepsin D activity (as measured against hemoglobin, pH 3.2) in each viscometer. When this is done, it is seen that the cartilage extract has a wider range of activity and is quite a bit more active at pH 5.5–6.5 than is purified cathepsin D. Recently, Woessner (18) has shown that cathepsin D from bovine uterus can be purified to the point where there is no action on PGS at pH's greater than 5.5. The preparation used for Fig. 1 still had residual activity at pH 7.

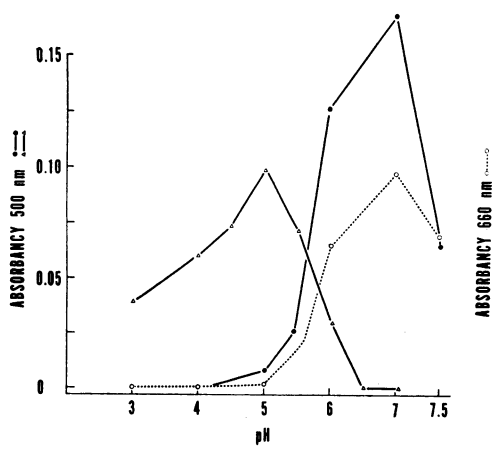

FIGURE 2 pH profile of histone digestion. ●, release of arginine by crude patellar extract containing 0.05 U cathepsin D; ○, release of tyrosine by the same enzyme; △, release of arginine by 1.5 U bovine cathepsin D. The assay conditions are described under Methods. Each point is the average of three determinations.

It is known that pepstatin, a microbial inhibitor of pepsin (31), is also effective in inhibiting cathepsin D (32). The inhibitor also binds to cathepsin D at pH 7. When this inhibitor was tested with cartilage extract (Table I), it was found to inhibit PGS digestion at pH 5 quite strongly, but to have only a small effect on the digestion at pH 7. There appears to be some spontaneous breakdown of PGS in this system; hence blocking the bovine cathepsin D did not completely prevent the loss of viscosity. Further incubation out to 20 h allowed further breakdown by non-D activity, so the percentage of inhibition diminished.

Two points emerge from this study. Cartilage extract activity at pH 5 is not due solely to cathepsin D; there seems to be a second enzyme participating in PGS digestion at this pH. Secondly, most of the activity at pH 7 is insensitive to pepstatin and is therefore attributed to an enzyme which is not cathepsin D. Similar results were found with EPP, a potent inhibitor of

pepsin (33); hemoglobin digestion at pH 3.2 was completely blocked, PGS digestion at pH 5 was blocked only partially, and there was only slight inhibition at pH 7. Again, the cartilage extract was less inhibited at pH 5 than the purified cathepsin D, suggesting a second enzyme acting at acid pH. Finally, highly purified bovine cathepsin D preparations were obtained which had no action at pH 7. Therefore, the cartilage contains at least one additional protease which acts at neutral pH. The action of this second enzyme may extend down to pH 5 or there may be a third enzyme acting at this acid pH.

When purified calf thymus histone was used as a substrate, the human cartilage extract digested the histone optimally at pH 7, both as measured by arginine or tyrosine release (Fig. 2). The small amount of cathepsin D in the extract (0.05 U) produced only a trace of activity on the histone at pH 5. 30 times this amount of highly-purified cathepsin D produced a prominent peak at pH 5, but had no effect on histone at neutral pH (Fig. 2). Pepstatin (1 μg) completely inhibited the digestion of histone at pH 5 by all of these preparations but had no effect on the digestion at neutral pH.

When azocasein was used as a substrate, the cartilage extract gave a broad activity peak between pH 6 and 8.5, as measured by either the release of azo dye or tyrosine (Fig. 3). Although this preparation contained only half as much cathepsin D (0.025 U) as the one used for histone digestion, it digested the casein to a greater extent than it did histone at pH 5–5.5. Pure cathepsin D did not digest casein at all at neutral pH. The activity curve shows two maxima at 5.5 and 3.5. Pepstatin (Fig. 3) did not inhibit the activity of the patellar extract at pH 7 and 8.5, but it reduced the peak at pH 6. Pepstatin inhibited all the activity of cathepsin D.

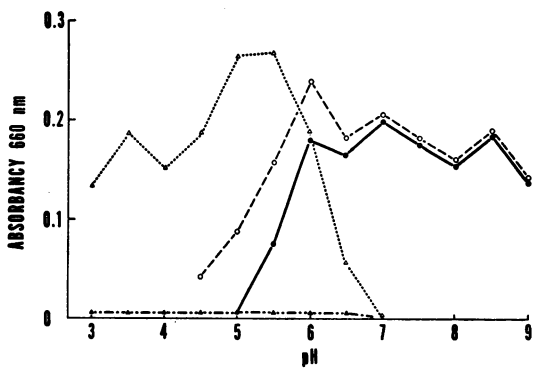

FIGURE 3 Azocasein digestion as a function of pH. △, release of tyrosine from 1% azocasein by 0.35 U purified bovine cathepsin D acting for 2 h; ▲, the same, plus 1 μg gram of pepstatin; ○, crude patellar extract containing 0.025 U cathepsin D; ●, the same, with pepstatin. Each point is the average of three determinations.

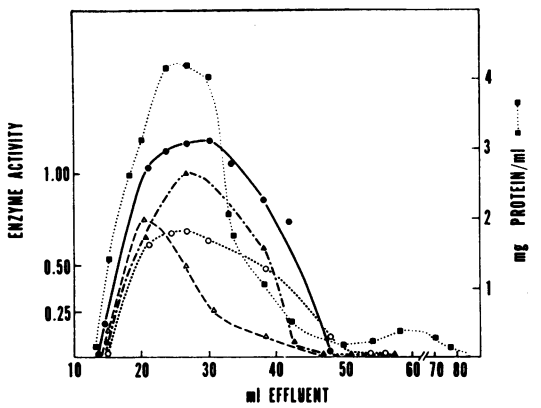

FIGURE 4 Chromatography of patellar extract on Sephadex G-100 as described in Methods. ●, cathepsin D, 1 unit on the ordinate equals 1 cathepsin unit; ▲, casein-digesting activity, 1 unit = 0.08 absorbancy unit at 450 nm; △, proteoglycan digestion, 1 unit = 40% loss of initial specific viscosity; ○, histone digestion, 1 unit = 0.04 absorbancy unit at 660 nm.

Purification of cartilage proteases. Partial purification of the various proteolytic activities of human cartilage has been achieved by chromatography on Sephadex G-100 and DEAE-Sephadex. When chromatographed on Sephadex G-100, the various protease activities emerged near each other, but not exactly superimposed (Fig. 4). The different shapes of the curves suggest that distinct proteins are responsible for each activity. Stepwise elution of the proteins from DEAE-Sephadex gave four peaks of cathepsin D activity (Fig. 5). The bulk of the protein came out in the first 100 ml and beyond 330 ml (not shown). The neutral activities likewise appeared in several peaks in approximately the same

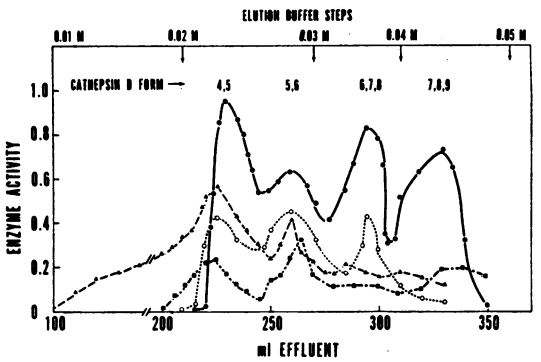

FIGURE 5 Chromatography of patellar extract on DEAE-Sephadex as described under Methods. Stepwise elution gave four peaks of cathepsin D activity. ●, cathepsin D, 1 unit = 0.25 D units; ▲, azocasein-digesting activity, 1 unit = 0.2 absorbancy unit at 450 nm; ○, histone-digesting activity, 1 unit = 0.2 absorbancy unit at 660 nm; ■, proteoglycan digesting activity, 1 unit = 50% loss of initial specific viscosity.

TABLE II

Partial Purification of Protease Activities in Patellar Extracts

	U/mg protein	Purification factor			
	Cathepsin D	Cathepsin D	Proteoglycan digesting activity	Caseinase	Histonase
Homogenate supernate	0.05	1	1	1	1
CPC treatment	0.21	4	2	2	2
G-100	0.57	12	4	3	3
DEAE-Sephadex	5.5	110	170	160	72
Disc electrophoresis	45.0	900	—	68	64

The purification of the proteoglycan digesting activity was measured by the percent original specific viscosity per milligram protein and that of the caseinase and histonase by the digest absorbancy values per milligram protein.

position as the first three cathepsin D peaks (Fig. 5). However, a large part of the first peak of the neutral activities appeared in the front of the first cathepsin D peak. As in the case of the G-100 chromatography the ratios of cathespin D and the neutral activities differed at all points along the curves.

Disc electrophoresis of each peak and identification of the active bands as described in Methods revealed that the cartilage cathepsin D existed in at least six multiple forms which migrated upon electrophoresis to the same positions as the bovine uterus cathepsin D multiple forms 4, 5, 6, 7, 8, and 9 (Fig. 6). The correspondence of the forms to the DEAE peaks is indicated on Fig. 5. In the bovine uterus, forms 4 and 5 predominate, whereas in the human cartilage forms 7, 8, and 9 were more pronounced. The other protease activities

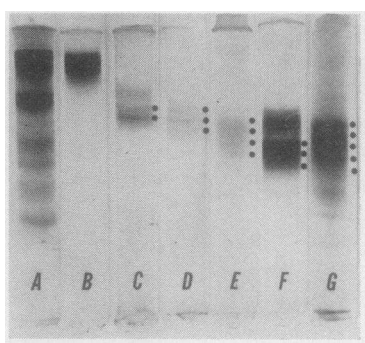

FIGURE 6 Disc-electrophoresis patterns prepared as described under Methods. (A) First peak of cathepsin D activity obtained from DEAE-Sephadex chromatography as illustrated in Fig. 5. A similar pattern was obtained with each of the four peaks from DEAE. The upper band (neutral protease activities) and the lower bands containing cathepsin D (marked ●) were eluted and reelectrophoresed to give patterns B–F. (B) Neutral protease band from pattern A. (C) Cathepsin D bands from the reelectrophoresis of the middle portion of pattern A. Two active bands (●) correspond to forms 4 and 5 of bovine uterus cathepsin D. (D) Cathepsin D forms found upon reelectrophoresis of the central region of the disc-gel material from the DEAE peak II (Fig. 5). Forms corresponding to 4, 5, and 6 are seen. (E) The corresponding pattern from DEAE peak III, showing forms 5–8. (F) Pattern from DEAE peak IV, showing forms 7, 8, and 9. (G) Pattern given by a combination of bovine uterine cathepsin D forms 5–10 as reference markers. Enzyme activities were identified by slicing duplicate unstained gels and eluting the activity.

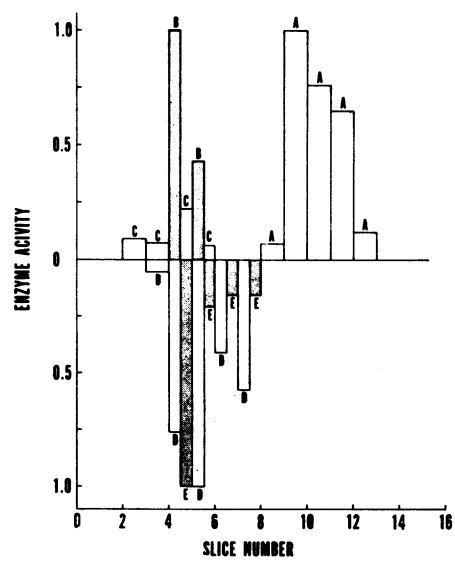

FIGURE 7 Protease activity in slices of disc-gel pattern A shown in Fig. 6. The gel was divided into 30 equal slices; the first 16 slices contained protease activities. Since some slices contained four activities, each slice is represented by two split bars extending above and below the 0 activity axis. (A) Cathepsin D activity, 1 unit = 0.3 D unit. (B) Casein digesting activity, 1 unit = 0.075 absorbancy units at 450 nm. (C) Cathepsin D-like activity occurring in small amount in the upper part of the gel. (D) Proteoglycan digesting activity, 1 unit = 0.07 absorbancy units from nonsedimentable uronic acid. (E) Histone digesting activity, 1 unit = 0.3 absorbancy units at 660 nm.

TABLE III

Digestion of PGS by Human Cartilage Extract at pH 7

Incubation mixture	Total digest, $S_{20,w}$*	CPC-TCA soluble fraction§ % total uronate‡	CPC-TCA soluble fraction§ $S_{20,w}$	CPC-TCA insoluble fraction % total uronate	CPC-TCA insoluble fraction $S_{20,w}$
PGS blank	18.0	8	9.5	89	17.3
PGS + extract:					
No addition	8.8	61	6.3	35	12.6
+ iodoacetate (25 mM)	8.0	60	5.0	36	12.0
+ cysteine (10 mM)	9.2	60	6.8	36	13.5
+ DFP (10 mM)	7.3	69	6.1	27	10.0
+ chloroquine	15.3	48	13.5	—	—

See Methods for details. Incubated 20 h at 37°C. The additions were preincubated with the enzyme + buffer solution for 15 min at room temperature. In the PGS blank, heat-denatured enzyme extract was used.

* Weight average sedimentation coefficient, determined at concentration of 0.04 mg uronate/ml.

‡ % of total uronate in 0.15 ml PGS (5 mg/ml), the amount of PGS used in the incubation mixture.

§ CPC, cetyl pyridinium chloride.

also appeared as multiple peaks, but it is not known if these peaks are isozymes or just different proteases with similar specificities.

A summary of the purification achieved for each of the proteolytic activities is given in Table II. The purification of the cartilage cathepsin D was increased greatly by cutting out the cathepsin D bands from the disc-electrophoresis gels (Fig. 6 C) since these bands moved to a region free from most of the inactive proteins. Unfortunately this was not the case for the neutral activities, since these bands migrated near the top of the column together with a lot if other proteins (Fig. 6 B). But neutral protease activity was well separated from cathepsin D activity. This is emphasized by Fig. 7 which shows the four enzyme activities in the top half of the disc gel illustrated in Fig. 6 A. Again, the impression is given that there are at least two distinct proteases acting at neutral pH.

The weight-average sedimentation coefficient of the 110-fold purified cathepsin D and of the 160-fold purified neutral caseinase from the DEAE peaks were 3.6 and 4.9, respectively. These S values are the average of three experiments, carried out by the microtransport method (24).

The 110-fold purified human cartilage cathepsin D activity, containing all the multiple forms, cleaved the B chain of insulin into the peptides shown in Fig. 8. These peptides correspond to cleavage at the peptide bonds Glu_{13}-Ala_{14}, Ala_{14}-Leu_{15}, Leu_{15}-Tyr_{16}, Tyr_{16}-Leu_{17}, Phe_{24}-Phe_{25}, and Phe_{25}-Tyr_{26}.

These are the same specific points of cleavage found for cathepsin D by Press, Porter, and Cebra (34) and Keilová (35) and used by them as the criterion for the rigorous identification of cathepsin D. The same splits are given by cathepsin D's from various sources (20, 30), including the multiple forms of the bovine uterus cathepsin D (20). The chief difference is the failure to observe a split at Phe_1-Val_2 in the present case. This is, in any event, a minor split (20).

When the cartilage extract acted on PGS at pH 7, the polymeric substrate with a weight-average sedimentation coefficient of 18S was degraded to fragments of 8.8S (Table III). Fractionation of these products with CPC and TCA yielded 61% soluble (average S = 6.3)

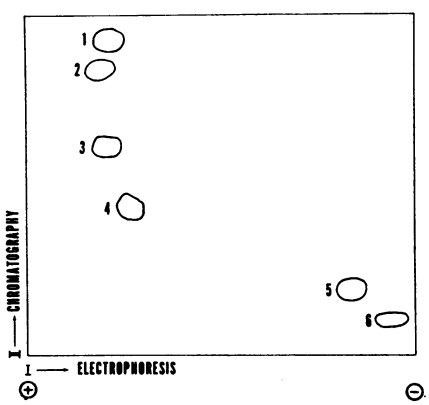

FIGURE 8 Fingerprint pattern of the peptides resulting from the digestion of the B chain of insulin by purified cathepsin D from human patella. Details are given under Methods. The peptides are identified as follows: (1) Leu_{15}, and Leu_{17} — Phe_{24}; (2) Phe_{25}, and Phe_1 — Glu_{13}; (3) Tyr_{16}; (4) Ala_{14}; (5) Phe_{25} — Ala_{30}; and (6) Tyr_{26} — Ala_{30}.

TABLE IV

Effect of Inhibitors on the Digestion of Various Substrates by Human Cartilage Extract at pH 7.0

Inhibitor	Substrate				
		Azocasein		Histone	
	PGS, viscosimetry	Release of tyrosine	Release of Azo dye	Release of tyrosine	Release of arginine
	%	%		%	
ε-Aminocaproic acid, 50 mM	0	0	0	0	0
Soybean trypsin inhibitor, 250 μg/ml	0	0	0	0	0
Cysteine, 10 mM	0	—	74	—	—
Chloroquine phosphate, 20 mM	60	68	50	66	—
Gold thiomalate, 2.5 mg/ml	0	0	0	69	66
Serum-human osteoarthritic, 1:10 dilution	—	69	67	60	—
Serum-normal 1:10 dilution	—	—	60	66	—
EDTA, 5 mM	50	68	63	98	—

Each evaluation is the mean of three to five experiments. Incubation was for 2 h at 37°C.

* Results expressed as percent inhibition; — not determined.

and 35% insoluble material (average S = 12.6). Using proteolytic activity purified by DEAE chromatography and elution from disc gels, the PGS was degraded to fragments of 3–6S. The products appeared to be large and heterogeneous. They did not pass through VisKing dialysis tubing.

The digestion of PGS at pH 7 was not affected by iodoacetate (25 mM), diisopropylfluorophosphate (10 mM), or cysteine (10 mM). However, chloroquine diphosphate (20 mM) strongly inhibited the digestion (Table III). Viscosimetric estimation of this inhibition yielded a value of 60% inhibition (Table IV). The activities against histone and casein may be due to distinct neutral proteases. The casein-digesting activity at pH 7 is inhibited by cysteine, whereas the histone-digesting activity is inhibited by aurothiomalate.

DISCUSSION

The present study was designed to explore the proteolytic activities of human articular cartilage with a view to explaining the degradation of the proteoglycan component of the cartilage matrix. The activities found included cathepsin D and neutral proteolytic enzymes digesting casein, histone and PGS. Cathepsin B and hyaluronidase were not present in cartilage as judged by the lack of effect of thiol reagents and the failure to produce dialyzable uronate at either pH 5 or 7.

As previously reported (12), the major cartilage protease with respect to hemoglobin and PGS digestion is cathepsin D. However, this predominance is seen only at acid pH. The identification of the cartilage activity as cathepsin D was based chiefly on the pH profiles for the digestion of these substrates in comparison with the pH profiles given by purified cathepsin D from bovine uterus. In the present paper this identification is proven conclusively by a study of the specificity of action of purified cartilage cathepsin on the B chain of insulin. It is further shown that the cartilage contains at least six multiple forms of cathepsin D, strengthening the resemblance to the well known cathepsin D of the bovine uterus.

Investigations from many laboratories have implicated cathepsin D in cartilage breakdown, even at neutral pH (14–16). However, recent studies with highly purified cathepsin D from bovine uterus have shown that this species of cathepsin D has no detectable action on proteoglycans at neutral pH (18, 19). The present findings confirm the conclusion that cathepsin D cannot be responsible for PGS digestion at pH 7. First, patellar extracts prepared at different times had different ratios of digestion of PGS at pH 7 and 5, indicating that the pH 7 activity was not proportional to cathepsin D content of the extracts. Highly purified bovine cathepsin D had no action at pH 7 on PGS preparations, casein or histone (Figs. 2, 3). Both pepstatin and EPP, potent inhibitors of cathepsin D, had little action on the neutral digestion of PGS, histone, or casein by patellar extracts (Table I). Finally, most of the neutral activity could be separated from cathepsin D by disc electrophoresis (Fig. 7).

While cathepsin D appears inactive at neutral pH, it is quite active at pH 5. In fact, this acidic activity overshadows the neutral activity. The role of cathepsin D in cartilage matrix breakdown must either be confined to intracellular digestion in the lysosomal system, or else there must be localized acidity around the chondrocyte. It has been suggested that anerobiosis might lead to lactic acid production by the chondrocytes (2, 36). How-

ever, microdeterminations of pH in cartilage fluid have yielded only neutral values (12).

A second acid cathepsin, cathepsin B, has been suggested as a possible candidate for cartilage degradation (37). Since this enzyme has a higher pH optimum than cathepsin D, its action might extend into the neutral region. However, we have not been able to detect cathepsin B activity on PGS in the previous (12) or present studies. There is no inhibition by iodoacetate or activation by cysteine (Table III). Dingle, Barrett, and Poole (38) found a small amount of iodoacetate-inhibitable activity in autolyzing human articular cartilage, but Ali and Evans (16) failed to find any cathepsin B in articular cartilage. It seems unlikely that cathepsin B plays an important role in articular cartilage metabolism. We were also unable to find any hyaluronidase-like activity at either acid or neutral pH. Cartilage extracts acting on PGS produced only large fragments ($S > 3$–6) with no diffusible uronic acid-containing products (Table III). Hyaluronidase has not been reported in any normal or osteoarthritic cartilages (2, 8), and the known hyaluronidase of tissue would not act at neutral pH (2). Chloroquine, which has no action on hyaluronidase (39), strongly inhibited PGS degradation in our studies (Table IV).

In view of the findings that cartilage readily undergoes autolytic release of proteoglycan at neutral pH (40), that this activity seems to be proteolytic (40), and that acid cathepsins seem insufficient to account for the effect, attention has been turned to neutral proteolytic activities. The digestion of PGS at neutral pH looks like a tail of activity on the peak seen at pH 5 (Fig. 1). However, the finding that more highly-purified cathepsin D had no activity at pH 7 points to the substantial activity of a second enzyme at pH 7.

To compare the neutral activity on PGS with neutral activities reported in the literature, two other protein substrates were investigated—histone and azocasein. There is some evidence that all three substrates may be digested by different enzymes. The three activities behaved differently on G-100 chromatography (Fig. 4) and DEAE-Sephadex chromatography (Fig. 5). The differences are not very pronounced, however, and the picture is complicated by the presence of several peaks of activity for each substrate. These peaks may represent multiple enzyme forms, or they may represent distinct enzyme species. While the neutral protease peaks parallel the cathepsin D peaks in these experiments, it is shown that cathepsin D in pure form has no action on these substrates (Figs. 2–3). The neutral enzymes were, as a group, well separated from cathepsin D by disc electrophoresis; they have not yet been resolved from each other. The azocaseinolytic activity, but not the PGS activity, was inhibited by cysteine (Table IV). Aurothiomalate inhibited the histonase activity but not the other two. EDTA had a different effect on each of the three enzymes. Obviously, detailed study is still required to determine the number of distinct proteases present in the cartilage.

We have not found any reports of neutral protease activity in articular cartilage which are sufficiently detailed to permit comparisons with the present activities. However, there have been suggestions that proteases from the synovial fluid or tissue or from leukocytes might initiate cartilage damage. The enzymes in cartilage appear to be different from these nearby proteases. Digestion of PGS was unaffected by DFP, indicating that the cartilage enzyme is distinct from one type of leukocyte protease (41). The cartilage activities do not resemble the enzyme of rheumatoid synovial tissue that is inhibited by cysteine (42), nor the enzyme of synovial fluid cells that is activated by cysteine (4). The histone-digesting enzyme of Davies, Rita, Krakauer, and Weissmann (43) appears unrelated to those in cartilage since it was inhibited by epsilon aminocaproate and soybean trypsin inhibitor. This last observation may explain why synovial inflammation induced by granulocyte lysosomal enzymes responds to epsilon aminocaproic acid treatment, whereas chronic cartilage degradation does not (44). In sum, it appears unlikely that the protease activities detected in cartilage originated in the synovium or leukocytes. It appears that neutral proteolytic activities are present in the cartilage; it is interesting to speculate that these activities are directly involved in the degradation of PGS, which is generally agreed to be one of the early steps in degenerative joint disease.

Human cartilage is thus found to contain two or more distinct proteases. Cathepsin D is quite prominent and may participate in intracellular digestion. It would be unlikely to have much effect on the extracellular matrix unless the pH fell to low levels. On the other hand, ample amounts of neutral protease are present: PGS, histone, and casein are all digested. There is some evidence that the PGS-digesting enzyme is distinct from the others, but further purification is required to settle this question.

ACKNOWLEDGMENTS

We are most grateful to Francisco Mueller and Rodolfo Denis for their assistance in the determination of the sedimentation coefficients and the digestion of the B chain of insulin.

This study was supported by Grants AM-08662, AM-05038, and AM-16940, National Institutes of Health and U. S. Veterans Administration, Miami, Fla.

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