# Metalloproteases of Human Articular Cartilage that Digest Cartilage Proteoglycan at Neutral and Acid pH

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ABSTRACT Extracts of human articular cartilage contain proteases capable of degrading the proteoglycan component of cartilage matrix at neutral and acid pH. These enzymes have been partially purified by ion exchange chromatography and characterized by disc electrophoresis, inhibition patterns, and action on proteoglycan. Three distinct metalloproteases are described. A neutral protease that digests proteoglycan subunit optimally at pH 7.25 has been purified up to 900-fold. It is strongly inhibited by *o*-phenanthroline, α-2-macroglobulin, and egg white, and to a lesser extent by D-penicillamine and EDTA. Inhibition by chelating agents is reversed by cobalt, zinc, and ferrous ions. Two acid metalloproteases, distinct from cathepsins B1, D, and F, digest proteoglycan subunit at pH 4.5 and 5.5. Both are inhibited by *o*-phenanthroline and activity is restored by cobalt, zinc, or ferrous ions. With electron microscopy, it was found that cartilage slices were depleted of ruthenium red-staining matrix proteoglycan after incubation in vitro with a partially purified cartilage extract at neutral pH. Sedimentation, gel chromatography, sodium dodecyl sulfate-gel electrophoresis, and immunodiffusion studies of digests of isolated proteoglycan fraction produced by the partially purified cartilage extract at neutral and acid pH confirmed that the cartilage enzymes act only on the protein component of proteoglycan subunit, producing fragments with 5 to 12 chondroitin sulfate chains. The link proteins were not digested.

## INTRODUCTION

In 1961, Lucy et al. (1) showed that vitamin A or hypoosmotic treatment of embryonic cartilage caused the release of a lysosomal acid cathepsin that digested the cartilage matrix. Since that time it has been widely held that this lysosomal acid cathepsin, now known as cathepsin D, is responsible for the degradation of cartilage matrix proteoglycan (2).

Recent work from our laboratory has called this assumption into question. The pH of the extracellular fluid of cartilage is normally about pH 7.3 (3), and we have shown that highly purified preparations of cathepsin D have no detectable hydrolytic effect on cartilage proteoglycans above pH 6 (4, 5). Thus, while human articular cartilage is rich in cathepsin D (3), the action of this enzyme must be limited to proteoglycans taken into digestive vacuoles or immediately at the cell surface, where an acid pH might be produced at the expense of metabolic energy. Moreover, we have shown (6) that human articular cartilage contains proteolytic activity at neutral pH, which could account for endogenous cartilage matrix degradation under physiological conditions.

The neutral protease activity has been studied with respect to its action on the proteoglycan subunit (PGS).[1] This subunit consists of a linear core protein with covalently attached side chains of chondroitin sulfate and keratan sulfate; it has a mol wt of about 2,500,000 daltons (7). The subunits assemble into large proteoglycan complexes by joining noncovalently to a hyaluronic acid chain; this binding is enhanced by the cooperation of

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[1] *Abbreviations used in this paper:* CM, carboxymethyl; EGTA, ethylene glycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetate; GPL, glycoprotein link fraction; HuCE, human cartilage extracts, combined fraction from CM-Sephadex chromatography; PGS, proteoglycan subunit; P, protease; SDS, sodium dodecyl sulfate; TLCK, tosyllysyl chloromethyl ketone; TPCK, tosylamide phenylethyl chloromethyl ketone; ZPCK, carbobenzyloxy phenylalanyl chloromethyl ketone.

two link proteins (7). The action of proteases on the subunit is to digest the protein core, producing peptide fragments with polysaccharide chains still attached.

The present study was undertaken to learn more about the proteases of human cartilage responsible for the degradation of PGS at neutral pH. Sufficient purification has been achieved to permit the demonstration of a metal-dependent protease that appears to be distinct from histone- and casein-degrading neutral proteases also present in the cartilage. In addition, two new acid proteases that digest proteoglycan have been discovered.

## METHODS

*Assay methods.* Methods have previously been published for the assay on a microscale of hemoglobin digestion by cathepsin D (3), and casein and histone digestion at neutral pH by cartilage extracts (6).

Proteoglycan subunit (PGS) and glycoprotein link fraction (GPL), the fraction containing link proteins, were prepared from bovine nasal cartilage by the method of Hascall and Sajdera (8). Digestion of PGS was quantitated in two ways. In the first method, PGS solutions were mixed with enzyme (0.03 ml) in a microviscosimeter (total volume 0.15 ml), as described previously (3). The decrease in viscosity was found to obey second-order kinetics, as also noted previously (4). A second-order rate constant is obtained by use of the relation $t^{-1}(\eta_0 - \eta_t)/(\eta_0 - \eta_\infty)$ in units per hour where $\eta$ is the flow time of the solution at times 0, $t$, and complete digestion. For example, when the change from $\eta_0$ to $\eta_\infty$ is 50% complete in 1 h, the rate constant $K = 1$ and when 75% complete, $K = 3$. The second method of measuring PGS digestion has recently been published (9). This involves enzymic digestion, the formation of a cetylpyridinium chloride complex with PGS, the release of degradation products by adding trichloroacetic acid, and the quantitation of these products by determining their uronic acid content. This assay is log-linear with respect to enzyme concentration, necessitating a correction before two levels can be directly compared. An absorbancy reading of 0.3 was selected as an arbitrary base point for comparisons and was defined as 0.3 enzyme U. Readings above or below 0.3 were converted to enzyme U by use of the published calibration curve (Fig. 4 of ref. 9). For example, an absorbancy of 0.40 is produced by twice the amount of enzyme that yields 0.30. Therefore, an absorbancy reading of 0.40 would be converted to 0.60 enzyme U to reflect this proportionality.

Lysozyme activity was assayed by the method of Litwack (10), with *Micrococcus lysodeikticus* cells (Worthington Biochemical Corp., Freehold, N. J.). The reaction volume was reduced by a factor of 20 and the absorbancy change was followed at 645 nm in a 0.4-ml cuvette.

*Enzyme purification.* 400 human patellae were removed at autopsy from male patients, age 40–70 yr, with no recognizable joint disease. The patellae were placed immediately on ice, trimmed, washed with ice-cold saline solution, and frozen until used. The crude enzyme extract was prepared from four batches of 100 patellae, which yielded 325 g of dissected cartilage in each batch. The dissected cartilage was finely diced and soaked overnight at 4°C in 5 mM phosphate buffer, pH 8.0, 3 ml/g tissue. This suspension was then homogenized with a VirTis Model 40 homogenizer (VirTis Co., Inc., Gardiner, N. Y.) for 5 min at ½ speed and 10 min at ¾ speed in an ice bath. The homogenate was centrifuged at 105,000 *g* for 30 min. The supernate was set aside and the pellet was frozen and thawed four times in a dry ice-acetone bath. This pellet was then extracted four more times, being homogenized in fresh buffer each time, and centrifuged as before. The combined five extracts of one batch were lyophilized, redissolved in a small volume of buffer, dialyzed against 5 mM phosphate buffer, pH 7.0, and centrifuged. A pellet remained that, when resuspended, showed no neutral protease activity and was discarded. 200 U of penicillin and 250 μg streptomycin were added per milliliter of supernate, and the preparation was stored at 4°C.

Each of the four batches was separately chromatographed on a 5 × 40-cm DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) column equilibrated with 5 mM sodium phosphate buffer, pH 7.0. After stepwise elution with increasing buffer concentration, three distinct peaks (labeled DEAE-I, II, and III) were separated, dialyzed against 5 mM phosphate to remove excess salt, and lyophilized. The fourth peak was not used in these studies due to its low specific activity. The four lyophilized preparations of peak I from DEAE-Sephadex were combined, dissolved in 40–45 ml phosphate buffer, and dialyzed against 25 mM sodium acetate buffer, pH 6.3. This material was then applied to a 2 × 85-cm column of carboxymethyl (CM)-Sephadex C-50 equilibrated with the same buffer. Elution followed with a series of gradients of gradually increasing acetate concentration. Active fractions of the eluate were combined, dialyzed versus 5 mM phosphate, pH 7.0, and lyophilized. This process was repeated with DEAE-II and III.

*Inhibitor studies.* All inhibitors were preincubated with the enzyme preparation at room temperature and pH 7 for 15 min before adding the substrate. Pepstatin (a generous gift of Dr. S. Itakura, Banyu Pharmaceutical Co., Okazaki, Japan) was dissolved in ethanol (1 mg/ml) and 2 μl of this was added to 200 μl incubation mixture. Ovomucoid was obtained from Pentex Biochemical, (Kankakee, Ill.), ethylene glycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetate (EGTA) from Sigma Chemical Company (St. Louis, Mo.), *o*-phenanthroline hydrate from K and K Laboratories, Inc. (Plainview, N. Y.), D-penicillamine and phenylmethane sulfonyl fluoride from Calbiochem (San Diego, Calif.), tosyllysyl chloromethyl ketone (TLCK) and tosylamide phenylethyl chloromethyl ketone (TPCK) from Nutritional Biochemicals Corp. (Cleveland, Ohio), and bovine nasal chondroitin sulfate from Mann Research Laboratories, Inc. (N. Y.). Carbobenzyloxy phenylalanyl chloromethyl ketone (ZPCK) was a gift from Dr. A. Janoff, Stony Brook, N. Y., Trasylol from Dr. K. Kuettner, Chicago, snake venom inhibitor from Dr. H. Tschesche, Munich, and human α-2-macroglobulin from Dr. A. J. Barrett, Cambridge, U. K. Egg white was removed from a fresh chicken egg, diluted 1:3 with pH 7.25 phosphate buffer, and centrifuged. Phenanthroline was dissolved first in 0.05 ml ethanol, then made to 1 ml with $H_2O$ to form a fine suspension. TPCK was dissolved in alcohol and ZPCK in dimethyl sulfoxide.

*Electron micrography.* Cartilage slices (500-μm thick) were obtained from sagittal sections of the growth plate of the tibia of a rachitic rat. One group of slices was incubated for 20 h in a solution of 0.05 M Tris-HCl, pH 7.4, 1.35 mM $CaCl_2$, 1.95 mM $Na_2HPO_4$, and 0.15 M NaCl. A second group was incubated in 0.5 ml of the same solution, to which was added 0.6 U of the human cartilage neutral protease from a combination of the CM-fractions (human cartilage extracts [HuCE] described in Results).

After incubation, the slices were stained for proteoglycan with ruthenium red, fixed in glutaraldehyde at pH 7.4, then postfixed in osmium tetroxide. The tissues were embedded in Epon (Shell Chemical Co., N. Y.) and sectioned at 600Å. Sections from the center of the 500-μm block were selected to determine enzyme penetration. Micrographs were made at a magnification of 12,000 with a Philips 300 electron microscope (Philips Electronic, Mount Vernon, N. Y.).

*Sedimentation studies.* A mixture of 0.15 ml PGS (5 mg/ml in 0.05 M phosphate buffer, pH 7.25) and 0.01 ml HuCE (0.6–15 μg protein/ml) was incubated at 37°C for 0.5–20 h. The reaction was stopped by chilling and dilution with 5 ml of 0.15 M KCl. The weight-average sedimentation coefficient of the digest was determined to ±0.5 S by the microtransport method of Pita and Müller (11) with previously described modifications (3).

*Sepharose gel chromatography.* PGS, 12 mg/ml in 0.15 M Tris-HCl, pH 7.4, or in 0.1 M acetate, pH 5.0, was incubated at 37°C with HuCE, 7–40 μg protein/ml, in the presence and absence of pepstatin, 20 μg/ml. Portions were removed after 0.5, 1, 2, and 4 h incubation and immediately frozen at −80°C. Several drops of toluene were added and the incubation was continued up to 20 h, when the remaining digest was frozen.

Portions of the thawed digests of PGS (0.9 ml) were applied to a 2.5 × 60 cm column packed with Sepharose 4B (Pharmacia Fine Chemicals). The column was eluted with 0.2 M NaCl at a flow rate of 20 ml/h at room temperature. The 2-ml fractions collected were tested for the presence of chondroitin sulfate by the carbazole reaction (12). The void volume, $V_o$, was determined by the elution maximum of PGS; the total column volume, $V_T$, was determined from the elution maximum of glucuronolactone. The elution volume of free chondroitin sulfate chains, $V_{cs}$, was determined with a sample purified by DEAE-cellulose chromatography (13).

Pooled eluates from the fractions indicated in Fig. 2 were dialyzed against distilled water and lyophilized. The average molecular weight of the fragments in these pooled fractions was determined by equilibrium sedimentation in the Spinco Model E ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) by the Yphantis method (14), closely following the speeds, solvents, and concentrations described by Heinegård and Hascall (15). The weight-average molecular weight was determined with the computer program of Small and Resnick (16) by averaging over the whole cell and by extrapolation of the number-average weight to the bottom of the cell. The values obtained by the two methods agreed to within a few percent and were averaged. Plots of the averaged molecular weights at the various concentrations were extrapolated to zero concentration to give the reported weight-average molecular weight of the Sepharose 4B eluate pools.

*SDS electrophoresis.* PGS, 10 mg/ml, and GPL, 2.5 mg/ml, in 0.15 M Tris-HCl, pH 7.4, or 0.1 M acetate, pH 5.0, were incubated with HuCE, 7–20 μg protein/ml, at 37°C for 0.5–20 h. Polyacrylamide gel electrophoresis with SDS was performed as previously described (17), except that the samples incubated at pH 5 were neutralized by adding an SDS solution with twice the usual concentration of pH 6.8 phosphate buffer. Gels were stained with Coomassie blue, 0.25% in water, and destained with 7% acetic acid.

*Immunodiffusion.* PGS, 10 mg/ml, or hyaluronidase-treated GPL, 2.5 mg/ml, in 0.15 M Tris-HCl, pH 7.4, or 0.1 M acetate, pH 5.0, was incubated with HuCE, 7–20 μg protein/ml at 37°C for 0.5–20 h. Hyaluronidase digestion was performed by incubating GPL or PGS at the above concentrations with 75 USP U/ml of bovine testicular hyaluronidase (HSEA, Worthington Biochemical Corp.) at 37°C for 6 h. Immunodiffusion was performed in 0.6% agarose at pH 8.6 as previously described (17, 18). The rabbit antisera to PGS and GPL were the same reagents used in previous studies (13, 17, 18). They were concentrated three times by vacuum ultrafiltration and absorbed with lyophilized bovine serum before use. The keratan sulfate and chondroitin sulfate-fragment fractions were prepared by trypsin-chymotrypsin digestion of PGS, followed by DEAE-cellulose chromatography. The detailed preparation procedure and the chemical and immunological properties of these fractions have been described in detail (13).

## RESULTS

*Purification of neutral protease.* The purification of neutral protease acting on proteoglycan is summarized in Table I. The initial extraction produced a large volume with low enzyme activity. The extracts were concentrated and applied to a DEAE-Sephadex column. This column bound proteoglycans firmly while the less tightly absorbed protease activity was eluted by increasing buffer concentrations.

The neutral proteoglycan-degrading activity eluted from the DEAE column in four distinct peaks (Fig. 1). Each peak also contained protease activity against histone and casein, as reported previously (6). The proportions of these three proteolytic activities varied considerably at different points of the elution pattern, suggesting that they may be due to distinct proteases. The behavior of a highly cationic protein, lysozyme, and an enzyme with known multiple forms, cathepsin D, is shown in Fig. 1 for comparison.

The DEAE fractions were combined into three pools corresponding to the first three elution steps, as indicated along the top margin of Fig. 1, and were labeled DEAE-I, II, and III. The fourth peak was not used in the present studies because of its low specific activity. Less than 2% of the total protein applied to the column emerged with the enzymes. The bulk of the protein in the cartilage extracts was not eluted until higher buffer concentrations were used (not shown) and the proteoglycan did not come out until the buffer reached 1 M.

*Acid protease activity.* When the crude extracts of cartilage were chromatographed on DEAE-Sephadex (Fig. 1), cathepsin D activity was found in all of the fractions, although it was most pronounced in DEAE-II and III. To determine whether all the acid proteolytic activity was due to cathepsin D, the DEAE fractions were assayed on PGS substrate over a range of pH's, with the addition of pepstatin to block all activity of cathepsin D. There are two distinct peaks of proteolytic activity under these conditions: one with a pH optimum near 4.5 and the second between 5.5 and 6.0 (Fig. 2). These activity peaks were not affected by dithiothreitol or iodoacetic acid, indicating that they are not due to

TABLE I

*Purification of Neutral Protease that Digests PGS, Uronate Assay, 30-min Incubation*

| Step | Volume | Units* | Protein | Specific activity | Purification | Yield |
|---|---|---|---|---|---|---|
| | *ml* | *U/ml* | *mg/ml* | *U/mg* | | |
| Combined extracts | 20,000 | 0.6 | 3.55 | 0.17 | 1 | 100 |
| Combined extracts lyophilized and redissolved | 1,500 | 10.54 | 13.83 | 0.76 | 4.5 | 132 |
| DEAE lyophilized | | | | | | |
| Peak I | 45 | 102.4 | 1.76 | 58.2 | 342 | |
| Peak II | 45 | 55.6 | 1.47 | 37.8 | 222 | 87 |
| Peak III | 40 | 82.0 | 2.19 | 37.4 | 220 | |
| CM lyophilized | | | | | | |
| Peak I | 40 | 70.2 | 0.45 | 156.0 | 920 | |
| Peak II | 40 | 36.9 | 0.38 | 97.1 | 570 | 70 |
| Peak III | 30 | 105.3 | 0.86 | 122.4 | 720 | |
| Peak IV | 40 | 24.0 | 0.45 | 53.3 | 310 | |

* As defined in Methods.

cathepsin B1. Since these two peaks do not seem to correspond to known cathepsins, we have provisionally designated them as protease (P) 4.5 and P 5.5. The two peaks were also present in DEAE-II (not illustrated).

*Chromatography on CM-Sephadex.* Each of the three DEAE fractions was chromatographed on CM-Sephadex columns, as illustrated in Fig. 3. DEAE-I produced a single major peak of neutral activity, designated CM-I, and a minor peak, CM-VII, which emerged later. DEAE-II and DEAE-III both gave peaks of neutral activity (CM-II and CM-III) in the same position as CM-I. DEAE-III also gave a second peak (CM-IV) in advance of the others. The four fractions from CM-Sephadex that contained significant neutral activity were numbered I–IV; their specific activities and yields are summarized in Table I. In each case the specific activity was increased two to fourfold by the CM step. When the CM-Sephadex fractions were assayed for acid protease activity in the presence of pepstatin to block cathepsin D, the new acid protease activities were found in every case to superimpose on the peaks of neutral protease activity. In addition, CM-fractions IV and VI were rich in these activities.

*Disc electrophoresis studies.* The individual CM-fractions I–VI were examined by polyacrylamide disc electrophoresis at pH 8.9. Each fraction contained at least two or three bands, and some single disc bands contained both acid and neutral activity. This survey indicated that there were several active forms of each of the proteases and that only small charge differences existed among the various forms. Preliminary study of the fractions by gel filtration on Bio-Gel P-60 (Bio-Rad Laboratories, Richmond Calif.) indicated that all of the enzyme forms had apparent molecular weights below 35,000 and that acid and neutral activities were not readily separable. This similarity in charge and size led us to conclude that it would be impossible to achieve complete resolution of the enzymes with the amount of cartilage extract available. Further experimentation followed two paths. A few experiments were performed on the individual CM-fractions. The remainder of the six CM-fractions were then combined to provide a preparation designated "human cartilage extract" (HuCE). This preparation was 400-fold purified with respect to neutral protease activity and was also enriched in acid protease activity. This was used for most of the following studies.

The HuCE preparation was subjected to disc electrophoresis for a prolonged time to enhance separation of the bands, since the proteins of interest move slowly at pH 8.9. The gels were sliced and eluted in buffer to recover the enzymatic activities (Fig. 4). Six of the nine bands had proteolytic activity. Cathepsin D activity moved completely off the gels. Neutral proteoglycan-degrading activity was found in bands 2, 5, 7, and 8 (J, K, L, and M: Fig. 4). Histonase and caseinase activities (not illustrated) were found only in the region of bands 1–2; the neutral activity in bands 5, 7, and 8 is free of these presumed contaminants. It should be noted that recoveries of neutral activity from the polyacrylamide gel are not quantitative.

In evaluating the results at pH 5.5, a problem arises in that the P 5.5 enzyme was never isolated in sufficient amount or purity to obtain a pH-activity curve. If this curve is fairly broad, activity of P 5.5 might still be appreciable at pH 7.25 or 4.5. By the same token, P 4.5 or the neutral protease might still have residual activity at pH 5.5. For this reason we identify only disc bands 1 and 7 as containing P 5.5 (E and G in Fig. 4); the activity at pH 5.5 seen in bands 3 and 8 (F and H in

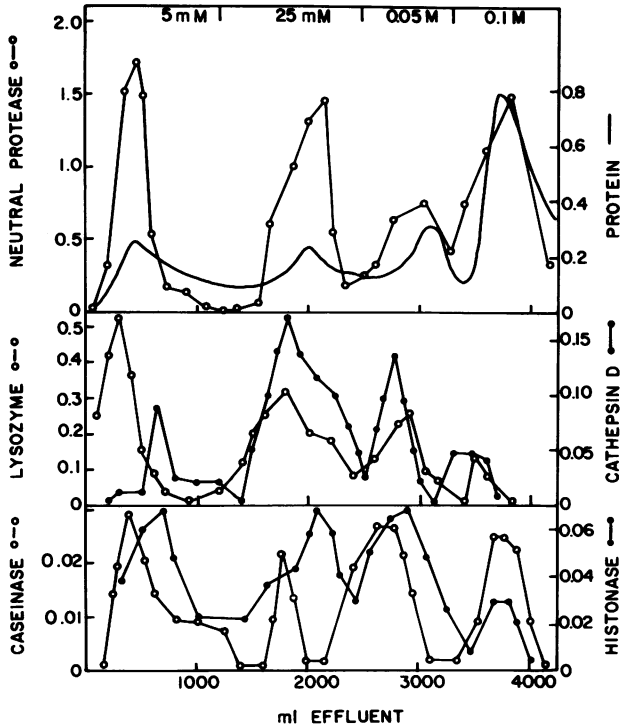


FIGURE 1 Chromatography of crude patellar extract on DEAE-Sephadex. Concentrated extract (375 ml) was dialyzed versus 5 mM phosphate buffer, pH 7.0, and applied to a 2 × 85 cm column of DEAE-Sephadex A-50 equilibrated with the same buffer. Elution was by stepwise increases in the molarity of the phosphate buffer, pH 7, as indicated at the top of the figure. In the top panel protein is shown as measured by absorbancy at 280 nm. Neutral protease activity on PGS at pH 7.25 as measured viscosimetrically at 37°C is plotted as the second-order rate constant ($h^{-1}$). The other enzyme activities are given in absorbancy units at 645 nm (lysozyme), 450 nm (caseinase), and 660 nm (cathepsin D and histonase). Four pools of eluate were collected corresponding to the four elution steps. These pools were labeled DEAE-I through IV.

Fig. 4) may well be residual activity of P 4.5. On the other hand, the considerable amount of P 5.5 in band 1 may be responsible for the apparent activity at pH 4.5 and 7.25 associated with this band (A and I in Fig. 4).

It is concluded that neutral protease activity is in bands 2, 5, 7, and 8; P 5.5 activity is in bands 1 and 7; and P 4.5 activity is in bands 2, 3, 7, and 8.

*Inhibitor studies.* Inhibitor studies were conducted with the separate fractions I-VI from CM-Sephadex (Fig. 3). The inhibitors studied and their concentrations were phenylmethane sulfonyl fluoride, TLCK, TPCK (10 mM); ZPCK (3 mM); Trasylol, and trypsin inhibitors of soybean, lima bean, and snake venom (250 μg/ml); ovomucoid (5 mg/ml); pepstatin (10 μg/ml); and iodoacetamide (5 mM). None of these compounds produced significant inhibition of the digestion of PGS at pH 4.5, 5.5, or 7.25 by any of the six CM-fractions. The enzymes in question are probably not of the serine, sulfhydryl, or carboxyl types.

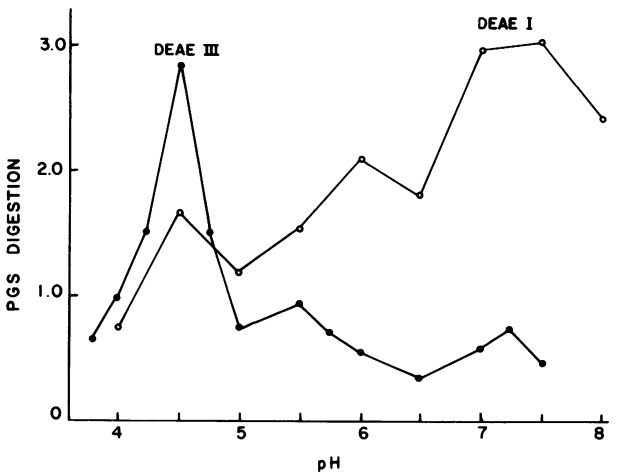


FIGURE 2 Digestion of PGS by DEAE fractions I and III as a function of pH. The activity was measured by viscosity change expressed in units of second-order rate constant ($h^{-1}$). The DEAE fractions are those described in Table I. The pH was controlled by 5 mM phosphate buffer over the range pH 6–8 and 0.05 M acetate buffer over the range pH 4–6. Pepstatin was present in all incubations at a concentration of 10 μg/ml to block cathepsin D activity.

The cartilage proteases appear to belong to the metalloprotease group, as indicated by the inhibitor studies of HuCE outlined in Table II. The neutral proteoglycan-degrading activity is inhibited to the extent of about 80% by penicillamine and 90% by phenanthroline, and to a lesser extent by EDTA and EGTA. Egg white and α-2-macroglobulin both inhibit about 85%.

Similar studies showed that the histone-digesting activity of HuCE was inhibited 100% by EDTA (10 mM), while the casein-digesting activity was inhibited only 40% by α-2-macroglobulin and 50% by *o*-phenanthroline.

TABLE II

*Effect of Inhibitors on PGS Digestion by HuCE*

| | pH | | |
|---|---|---|---|
| | 4.5 | 5.5 | 7.25 |
| | *Uninhibited enzyme activities** | | |
| No addition | 3.75 | 2.42 | 2.11 |
| | *% inhibition* | | |
| Egg white, 15 μl/ml | 85 | 90 | 86 |
| α-2-Macroglobulin, 250 μg/ml | 45 | 78 | 87 |
| Phenanthroline, 5 mM | 88 | 90 | 91 |
| Penicillamine, 20 mM | 10 | 63 | 78 |
| EDTA, 10 mM | 61 | 61 | 68 |
| EGTA, 20 mM | 37 | 40 | 55 |

* All assays were performed by the viscosity decrease method. Enzyme units were calculated as second-order rate constants per hour. Inhibition calculations were based on these units. Assays and blanks were run in duplicate.

The pH 4.5 protease shows a pattern of inhibition slightly different from that of the neutral activity (Table II). Phenanthroline and egg white inhibit strongly; and EDTA and EGTA, less strongly. However, α-2-macroglobulin inhibits weakly and penicillamine not at all at this lower pH. Possibly the acid pH prevents their effective interaction with the enzyme.

It is difficult to deduce the properties of the P 5.5 enzyme since it has not been separated from the other two protease activities. It seems safe to conclude that it is strongly inhibited by phenanthroline, egg white, and α-2-macroglobulin, and to a lesser extent by penicillamine (Table II).

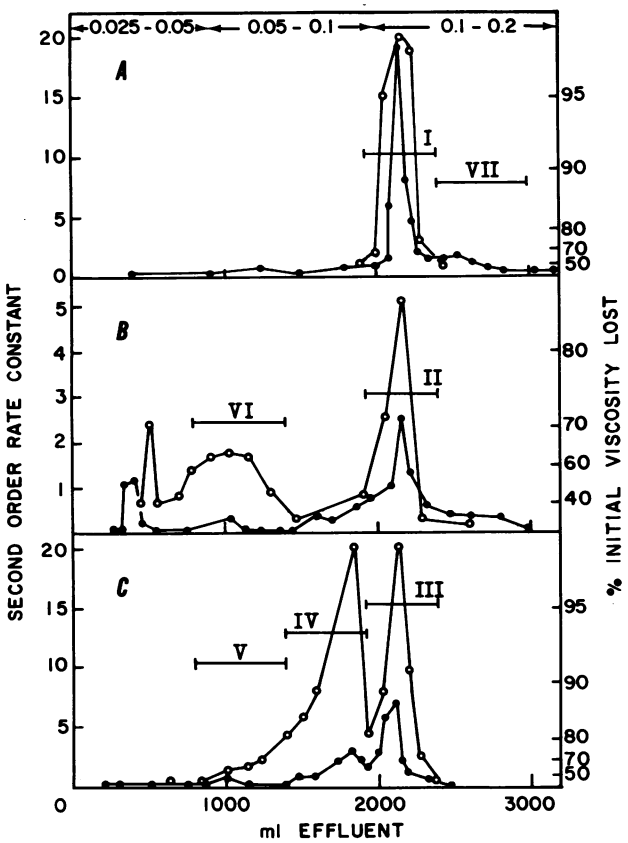


FIGURE 3 Chromatography of DEAE-fractions of patellar extract on CM-Sephadex. The fractions from DEAE chromatography and the CM-Sephadex column (2 × 85 cm) were both equilibrated with 25 mM acetate buffer, pH 6.3. The column was eluted with three linear gradients of increasing acetate buffer concentrations, indicated at the top of the figure. Panels A, B, and C show the elution patterns obtained upon chromatography of DEAE fractions I, II, and III, respectively. The closed circles (●) show PGS-digesting activity at pH 7.25 and the open circles (○) at pH 4.5. Activity at pH 5.5 paralleled that at 4.5, but was lower at all points. The assay was by viscosity changes; second-order rate constants are plotted. Pepstatin was added to all assays at pH 4.5 to block cathepsin D. Note the change of scale in panel B. Seven pools of active fractions were made and labeled CM-I to CM-VII, as indicated on the figure.

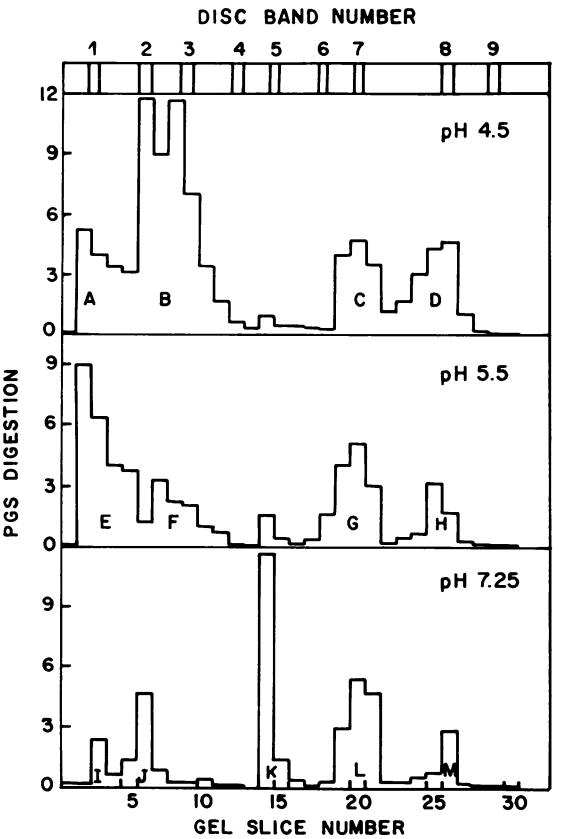


FIGURE 4 Enzymatic activity of disc electrophoresis bands. Samples of HuCE were subjected to disc electrophoresis in 7.5% polyacrylamide gel, 7-cm columns, by the standard method of Davis (19). Gels were developed until the bromphenol tracking dye reached the end of the column (about 60 min), and then electrophoresis was continued for an additional 50 min. Four samples were prepared. One was stained with Coomassie blue (20), and three were sliced into 30 uniform slices each. Corresponding slices were combined, eluted in 5 mM phosphate buffer, pH 7.0, and assayed for PGS-digesting activity at pH 4.5, 5.5, and and 7.25 by viscosimetric assay. The results are plotted as second-order rate constants $(24\ h)^{-1}$ in correspondence to the pattern of nine bands shown at the top of the figure. Pepstatin was added to all assays at pH 4.5 and 5.5.

The metal dependence of the proteoglycan-degrading activities of HuCE is confirmed by the studies outlined in Table III, which demonstrates the ability of metal ions to reverse the inhibition produced by phenanthroline and EDTA. Cobalt, zinc, and ferrous ions give fairly complete reversal of inhibition at all pH's in the case of phenanthroline. With EDTA only cobalt gives good reversal at pH 7.25; zinc and ferrous ions are less effective. Calcium, magnesium, and manganese do not reverse inhibition by either chelator, and calcium may even be somewhat inhibitory. When the enzyme is dialyzed extensively against distilled water, it loses neutral activity, probably by denaturation. Cobalt offers some

TABLE III

*Effect of Metal Ions on Reversal of Inhibition of PGS Digestion by Chelators*

| | Inhibition at pH | | |
|---|---|---|---|
| | 7.25 | 5.5 | 4.5 |
| | % | % | % |
| *o*-Phenanthroline | 87 | 83 | 80 |
| +$CoCl_2$ | 19 | 15 | 20 |
| +$ZnCl_2$ | 17 | 16 | 0 |
| +$FeSO_4$ | 19 | 10 | 6 |
| +$FeCl_3$ | 68 | 60 | 77 |
| +$CaCl_2$ | 88 | 81 | 89 |
| +$MgSO_4$ | 80 | 76 | 86 |
| +$MnCl_2$ | 89 | 64 | 84 |
| EDTA | 44 | 60 | 63 |
| +$CoCl_2$ | 7 | 15 | 5 |
| +$ZnCl_2$ | 27 | 33 | 20 |
| +$FeSO_4$ | 30 | 13 | 6 |
| +$CaCl_2$ | 66 | 55 | 75 |
| +$MgSO_4$ | 50 | 40 | 66 |
| +$MnCl_2$ | 45 | 49 | 63 |
| Dialyze vs. water | 83 | | |
| Dialyze vs. 2 mM $CoCl_2$ | 28 | | |
| Dialyze vs. 2 mM $ZnCl_2$ | 65 | | |

A pool of enzyme (HuCE) was mixed with chelating agent at a concentration of 4 mM and allowed to react for 15 min at room temperature. Metal salts were then added to a concentration of 8 mM for an additional 15 min. 3 vol of PGS solution was added (reducing final salt concentrations to 2 mM) and viscosimetric assays were performed at 37°C. Insoluble $FeSO_4$ was removed just before assay by centrifugation.

protection against this loss and zinc offers less. Neither ion could restore lost activity.

*Effect of neutral protease on cartilage.* When sections of rachitic rat cartilage were incubated with HuCE at neutral pH, matrix components staining with ruthenium red, representing for the most part proteoglycan, were depleted throughout the 500-μM thickness of the sections (Fig. 5). This indicates that the neutral enzyme can penetrate intact cartilage slices and digest the matrix proteoglycans in vitro, without being blocked by possible endogenous protease inhibitors in the cartilage.

*Effect of HuCE on isolated proteoglycan fractions: sedimentation studies.* The weight average sedimentation coefficients of PGS fragments produced by varying amounts of HuCE acting for varying lengths of time are summarized in Table III. With increasing amounts of HuCE and longer incubation periods, the sedimentation coefficient of the PGS digest progressively decreased, indicating that the average size of the proteoglycan fragments became smaller. However, even with large amounts of HuCE and prolonged incubation, the weight-average sedimentation coefficient was never smaller than the limiting value of 3S (Table IV).

*Sepharose gel chromatography.* When PGS was chromatographed on Sepharose 4B, it appeared in the excluded volume due to its large molecular weight and extended configuration. After incubation with HuCE (7 μg/ml) for 1 h at pH 7.4 or 5.0, the fragments were still too large to appear in the included volume of Sepharose 2B (Fig. 6A and B). After 20 h incubation, the resultant proteoglycan fragments eluted entirely within the Sepharose 4B-included volume (Fig. 6A, B). No uronic acid-containing material was found at the column volume, $V_T$, or at the position of single chains of chondroitin sulfate, $V_{cs}$; all of the uronic acid applied to the column is accounted for in the fractions illustrated in Fig. 6. The eluate fractions indicated in Fig. 6A (top scale) were pooled and the weight-average molecular weight of the fragments in each pool was determined by equilibrium sedimentation (Fig. 6A). These results indicate that HuCE degraded the core protein of PGS to peptide fragments with approximately 5–12 chondroitin sulfate chains, and that it did not degrade the polysaccharide chains.

PGS incubated at pH 5 with HuCE (7 μg protein/ml) was degraded more slowly when pepstatin was added to block cathepsin D action, as indicated by the elution of most of the proteoglycan fragments in the Sepharose 4B excluded volume after 4 h incubation (Fig. 6C). After 20 h incubation, the elution profile was essentially the same as without pepstatin. This indicates that the acid metalloproteases can digest PGS to at least the same extent as cathepsin D.

When the concentration of HuCE was increased to 40 μg protein/ml, some proteoglycan fragments small enough to elute in the Sepharose 4B-included volume appeared after 1 h of incubation. However, the elution profile after 20 h incubation at pH 7.4 was identical to that shown in Fig. 6A, and that obtained at pH 5 was only slightly shifted to the right as compared to Fig. 6B,

TABLE IV

*Sedimentation Analysis of PGS Digestion Products*

| HuCE | 0.5 h | 1 h | 2 h | 20 h |
|---|---|---|---|---|
| *μg protein* | *S* | *S* | *S* | *S* |
| 15 | — | — | — | 3.3 |
| 3 | 9.0 | 6.7 | 4.0 | 3.2 |
| 1.5 | — | — | — | 4.1 |
| 0.6 | 13.3 | 10.8 | — | 9.2 |
| 0 | — | — | — | 20.5 |

The values shown represent the weight average sedimentation coefficient of the digests obtained by the incubation of PGS with the indicated amounts of HuCE at pH 7.25 and 37°C. Portions were removed at intervals of 0.5–20 h for sedimentation analysis (11).

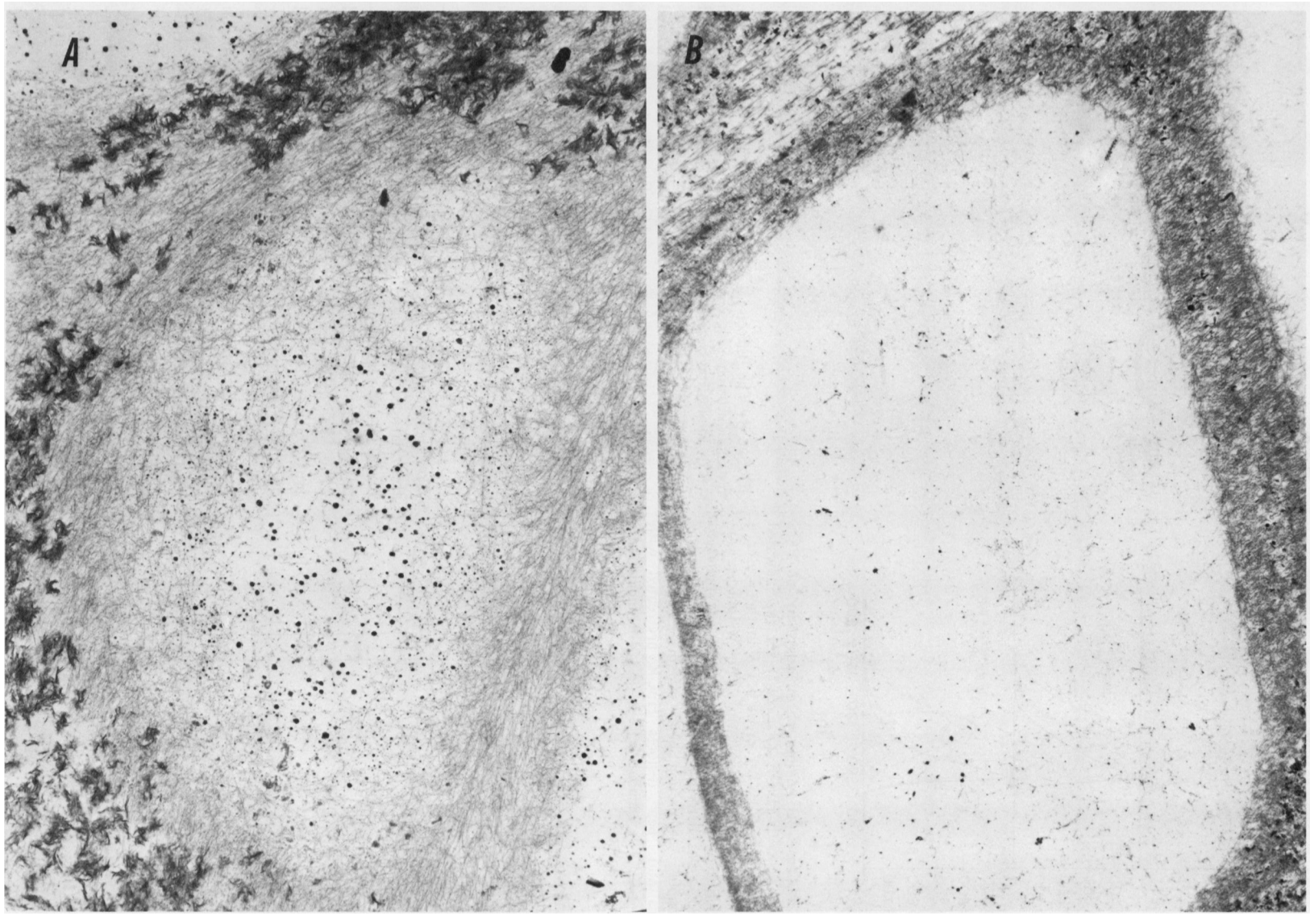


FIGURE 5 Electron micrographs of rachitic rat cartilage. (A) Control. (B) Treated 18 h with neutral protease as described in Methods. Note the marked diminution of ruthenium red-staining granules (black particles) in the central area overlying the chondrocyte, surrounded by denser septa.

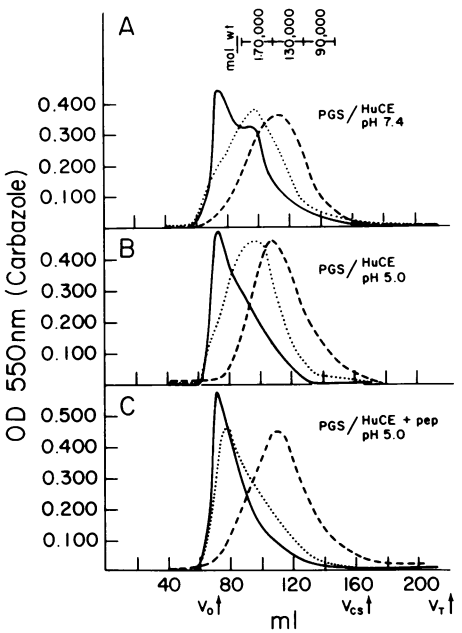


FIGURE 6 Sepharose 4B column chromatography of PGS incubated at 37°C with HuCE (7 μg/ml) for the indicated periods of time at pH 7.4 (A) or at pH 5 in the presence (B) or absence (C) of pepstatin. The weight-average molecular weight of the fragments eluting in the indicated pooled fractions was determined by equilibrium sedimentation. $V_{cs}$ denotes the elution peak of single chondroitin sulfate chains and "pep" denotes HuCE pretreated with pepstatin. ———, 1-h incubation; ....., 4-h incubation; - - - - - -, 20-h incubation.

with the peak of carbazole-reactive material eluting at 120 ml. These results agree with the sedimentation results in establishing the existence of an apparent limit to degradation, reached when the PGS fragments still consist of 5–12 chondroitin sulfate chains.

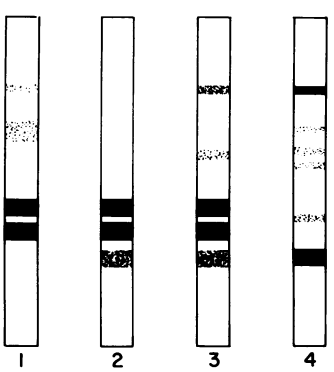


FIGURE 7 SDS-polyacrylamide gel electrophoresis of glycoprotein link fraction before and after incubation with HuCE for 20 h at 37°C and the indicated pH. The gels were stained for protein with Coomassie blue. 1. GPL; 2. GPL digested with HuCE at pH 5; 3. GPL digested with HuCE at pH 7.4; 4. HuCE.

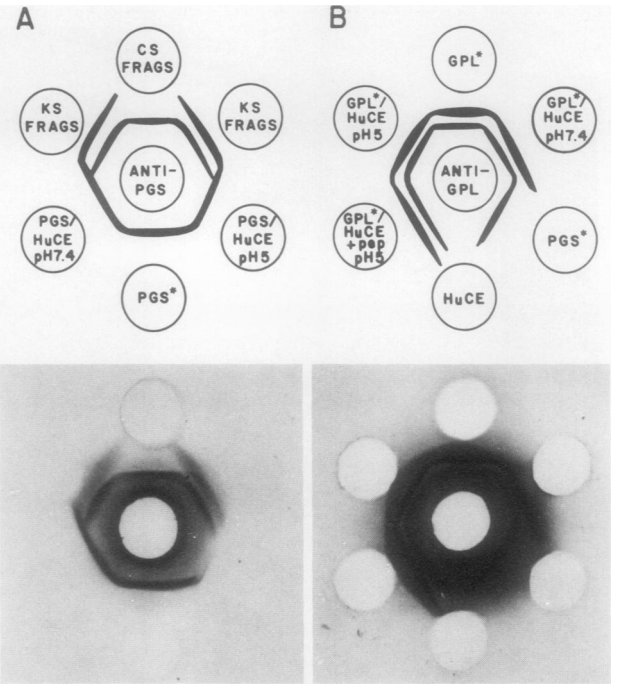


FIGURE 8 The effect of treatment with HuCE on the immunodiffusion reactivity of PGS (A) and GPL protein (B). Incubation was carried out at 37°C for 6 h at pH 7.4, or at pH 5 in the presence of or absence of pepstatin. * denotes hyaluronidase-treated proteoglycan fractions and "pep" denotes pretreatment of HuCE with pepstatin. The well labeled "CS frags" contained chondroitin sulfate-peptides and the well labeled "KS frags" contained keratan sulfate-peptides isolated from trypsin-chymotrypsin digests of PGS. PGS and "CS frags" were used at 10 mg/ml, GPL* and "KS frags" at 2.5 mg/ml. (The precipitin line at 4 o'clock in plate A became attenuated in the course of preparing the plate for staining. The diagram depicts the appearance of the unwashed plate.)

*SDS-polyacrylamide gel electrophoresis studies.* After digestion of GPL with HuCE at pH 5 or at pH 7.4, the characteristic migration positions of the two major protein bands of the proteoglycan link proteins on SDS-polyacrylamide gel electrophoresis were unchanged (Fig. 7). SDS-polyacrylamide gel electrophoresis of HuCE digests of PGS at acid and neutral pH yielded only very faint protein bands, not adequately distinguishable from those due to HuCE and PGS contaminants.

*Immunodiffusion studies.* Previous immunodiffusion studies have indicated that the PGS core protein has at least two antigenic determinants (13). Each determinant is present on separate fragments in the fraction of trypsin-chymotrypsin digest of PGS containing only keratan sulfate, whereas only one of the two determinants is present in the fragments in the chondroitin sulfate-containing fraction, as shown in the upper part of Fig. 8A. The reaction of antisera to PGS with PGS digested with HuCE at pH 7.4, and at pH 5 formed

single precipitin lines. That there is no crossing where this single line meets the two precipitin lines formed with the keratan sulfate-fragment fraction indicates that both core protein determinants are present in the one line. The formation of only a single precipitin line after digestion with HuCE suggests that the various antigenic fragments produced are all similar in their diffusion rate in agarose or that both core protein antigenic determinants are still present on the same fragment.

The immunodiffusion reaction of hyaluronidase-digested GPL with antisera to GPL yields two precipitin lines, the line nearer to the antigen well being related to the proteoglycan link proteins (15). Digestion of hyaluronidase-treated GPL with HuCE at pH 5 and pH 7.4 did not affect the immunodiffusion reaction with anti-GPL sera (Fig. 8B).

## DISCUSSION

If one studies the breakdown of proteoglycan by cartilage extracts or the autolytic breakdown of the matrix of cartilage slices in vitro, one finds that the degradative activity is very pronounced at pH 5 and relatively insignificant at physiological pH (21, 22). Such observations led to the conclusion that cathepsin D must be the major degradative protease in human articular cartilage (23). While this conclusion is valid, it has tended to draw attention away from other proteases that may be less important quantitatively, but more important physiologically.

The present study builds on our earlier observation (6) that human articular cartilage extracts display proteoglycan-degrading activity at neutral pH. This activity has been purified 300- to 900-fold in several fractions. The purest fractions are one-tenth as active (on a protein basis) as trypsin in digesting PGS. The extent of inhibition by the chelating agents, *o*-phenanthroline and D-penicillamine, indicates that most, if not all, of this activity is metal-dependent. It is possible that this activity is due to a single metalloprotease, with the four disc-electrophoresis bands (Fig. 4) representing isozymes.

In addition to the neutral proteoglycan-degrading activity, cartilage extracts also contain neutral proteases that digest histone and casein. Three lines of evidence suggest three distinct proteases for the three different substrates. First, it is possible to obtain fractions such as CM-II that have no activity on histone or casein, indicating the presence of a neutral protease relatively specific for proteoglycan. Second, studies carried out previously (6) showed that the histone-digesting activity was inhibited 70% by aurothiomalate, a compound with no effect on PGS digestion. Third, the present study shows that histone digestion is more completely inhibited by EDTA, and casein digestion is less completely inhibited by $\alpha$-2-macroglobulin and *o*-phenanthroline, than the proteoglycan-digesting activity. Final conclusions about the total number of proteases must await further purification.

There have been only a few previous reports of the presence in cartilage extracts (24, 25) or autolyzing slices (26–29) of matrix-degrading enzymes active at neutral pH. The present study appears to be the first to describe the partial purification of a neutral proteoglycan-degrading enzyme and its identification as a metalloprotease. Very few mammalian metalloproteases are known. The cartilage protease may be distinguished from leukocyte gelatinase (30), kidney brush border protease (31), and lens protease (32) on the basis of their published characteristics. Fibroblasts, cultured from rabbit synovial membrane, secrete a protease strongly inhibited by EDTA and phenanthroline (34); this may be related to the cartilage enzyme.

Two new acid proteolytic enzyme activities are clearly demonstrated (Fig. 2) when PGS digestion is carried out in the presence of pepstatin to block cathepsin D activity; these activities have been provisionally designated at P 4.5 and P 5.5. These enzymes are also metal-dependent and appear to occur in multiple forms (Fig. 4).

There have been numerous previous reports of acid proteolytic activity in cartilage, but all of these appear to be adequately explained by the presence of cathepsin D (4). The only exceptions are cathepsin B1, found in elastic cartilage by Ali and Evans (23), but absent or very low in human articular cartilage, and cathepsin F (34), also found in elastic cartilage. The two acid enzymes we report here are clearly distinct from cathepsin B1, which is sulfhydryl-dependent; from cathepsin F, which is not EDTA-inhibitable; and from cathepsin D, which is completely inhibited by pepstatin. Virtually nothing is known of acid metalloproteases. There is a human lens metalloprotease, but it is unaffected by *o*-phenanthroline (35).

Until now it has been assumed that the proteoglycan-degrading enzymes of cartilage extracts are proteases because hyaluronidase-like activity has been found to be absent from cartilage (36). Characterization of the products of HuCE digestion of PGS has provided evidence that this assumption is correct. After Sepharose gel chromatography of neutral or acid pH digests, all the uronic acid-containing material applied to the column was recovered in the eluates, but small uronic acid-containing fragments and single chondroitin sulfate chains were not detected. Thus, enzymes with hyaluronidase- or chondroitinase-like activity, or enzymes acting on the neutral sugar bridge linking chondroitin sulfate to the PGS core protein are not present in HuCE, and the ability of HuCE to degrade PGS must reside solely in its ability to disrupt the core protein. The enzymes are endopeptidases, not exopeptidases, since the latter

would give rise to single chains of chondroitin and keratan sulfate as products.

It is instructive to compare the effects of HuCE on cartilage proteoglycan described here with the results found in similar studies of the effects of human leukocyte granule extracts and two purified neutral proteases, an elastase and a chymotrypsin-like enzyme, isolated from these extracts (37). The leukocyte enzymes degrade link b and produce a fragment from the hyaluronic acid-binding portion of the PGS readily detectable by SDS-polyacrylamide gel electrophoresis. Furthermore, crude leukocyte granule extract and purified granule elastase are capable of degrading PGS to peptides with a single chondroitin sulfate chain (24). On the other hand, even after prolonged incubation of proteoglycan fractions with high concentrations of HuCE at acid or neutral pH, the two link proteins remain intact, a fragment from the hyaluronic acid-binding portion of the PGS is not clearly detectable, and only limited degradation of the chondroitin sulfate-containing portion of the PGS occurs, as indicated by the failure to lower the weight-average sedimentation coefficient below 3S and by the absence of fragments with fewer than five chondroitin sulfate chains noted upon gel chromatography. Apparently, the proteases in HuCE are able to cleave the PGS core protein at only a limited number of sites. Although HuCE is only partially purified, it seems highly unlikely that any individual cartilage protease could degrade proteoglycan more than the multiple proteases in HuCE acting simultaneously.

The demonstration of proteases, in normal cartilage, capable of digesting PGS molecules under physiological conditions may be of considerable significance for the eventual elucidation of the mechanisms of normal metabolic turnover of the matrix and its pathological destruction, as in osteoarthritis.

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