Purification of Cathepsin D from Cartilage and Uterus and Its Action on the Protein-Polysaccharide Complex of Cartilage*

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SUMMARY

Cathepsin D has been purified approximately 900-fold from two types of cartilage: elastic cartilage of young rabbit ears and hyaline cartilage from the legs of 12- to 13-day-old chick embryos. Cathepsin D accounts for most of the hemoglobin-digesting activity of chick cartilage; rabbit ear cartilage also contains a sulfhydryl-dependent acid cathepsin. The identification of the acid proteolytic activity of the cartilages as cathepsin D is based on the specificity of cleavage of the B chain of insulin. The molecular weight of the two enzymes, as estimated by molecular sieving, is about 40,000 to 43,000.

The two cartilage enzymes and cathepsin D from bovine uterus all digested the protein-polysaccharide complex of bovine nasal cartilage at pH 5. The digestion could be followed viscosimetrically and it obeyed a second order kinetic expression. The protein-polysaccharide complex was completely degraded to fragments that were larger than 10,000. This digestion was inhibited by e-aminocaproate and arginine (0.1 M), chloroquine and 3-indolepyruvate (0.02 M), and pepstatin (2 × 10⁻⁷ M).

Activity curves were measured as a function of pH; all three preparations of cathepsin D digested protein-polysaccharide complex optimally at pH 4. On comparing one hemoglobin-digesting unit of each enzyme, it was found that the cathepsin D from bovine uterus digested protein-polysaccharide complex twice as rapidly as the rabbit ear enzyme and 8 times as rapidly as the chick cartilage enzyme. At pH 7.2 digestion of protein-polysaccharide complex was quite variable, the chick preparation was completely inactive, and the other preparations had variable activities that were not in proportion to their hemoglobin-digesting activity. It was finally shown that cathepsin D is completely inactive at pH 7.2 and that protein-polysaccharide complex digestion at this pH is due to the action of a contaminant neutral protease.

The mechanisms by which cartilage matrix is broken down assume considerable importance in pathological disturbances such as osteo- and rheumatoid arthritis (1) and in normal processes of remodeling and calcification (2). The major extracellular components of the matrix are the collagen fibers and the protein-polysaccharide complexes. These two components are probably broken down by independent pathways. The collagen may be degraded by specific collagenases (3), whereas the protein-polysaccharide complexes could be attacked by a variety of proteases and polysaccharidases. The protein-polysaccharide complexes consist of large proteoglycan subunits linked into larger aggregates by an intermediate linking protein (4). Earlier efforts at extracting these complexes from cartilage resulted in a related fraction known as protein-polysaccharide-light fraction (5). Most workers have addressed themselves to the breakdown of the protein backbone of protein-polysaccharide complex or proteoglycan subunits. This backbone holds the side chains of chondroitin and keratan sulfates. The structure of this backbone is such that almost any protease of general specificity might be expected to digest it (6). The question, then, is which proteases are present in the cartilage and could account for the digestion of the complexes.

It is known that vitamin A, when administered in excessive amounts, will cause the loss of cartilage matrix from rabbit ears and cultured chick-embryo limbs (8). This effect of vitamin A is attributed to a rupturing of lysosomal membranes and the consequent release of lysosomal proteases (9). The Strangways group (10) extracted a protease from embryonic chick limb cartilage which had an acid pH optimum and was suggested to resemble cathepsin D. Ali (11), on the other hand, found an enzyme in rabbit ear cartilage which he believed to be cathepsin B. This enzyme was inhibited by arginine and chloroquine. More recently, Ali (12) has concluded that rabbit ear cartilage contains both cathepsins B and D, but that articular cartilage contains only cathepsin D. Dziewiatkowski (13) partially purified an acid cathepsin from calf costal cartilage. This enzyme has not been fully characterized, but it seems likely that it is also cathepsin D. The Strangways group has shown that the breakdown of embryo cartilage in vitro at pH 5 is strongly inhibited by antiserum to cathepsin D (14, 15) and the breakdown of human cartilage in vitro at pH 5 is inhibited by the specific inhibitor, pepstatin (16). These lines of evidence indicate that cathepsin
D may be one of the major proteases in the cartilage and that it can digest the matrix at acid pH.

However, it has been recognized that an enzyme requiring an acid pH of 5 may not function physiologically, particularly if it is in the extracellular matrix rather than in a digestive vacuole. This has led to studies of the upper limit of the pH range for the action of cathepsin D. The results found to date strongly suggest that cathepsin D does, in fact, have some effect on protein-polysaccharide complexes at pH 7. I reported such an effect in 1967 (17), and the Strangeways group has made a series of observations along these same lines. For example, Dingoe et al. (15) have shown that purified cathepsin D digests cartilage matrix at a significant rate at pH 7 and that both this digestion and also treatment: elastic cartilage from the ears of young rabbits and purified preparations of both types of cartilage were based on insulin. I set out to establish the presence of cathepsin D in the manner as the rabbit enzyme.

The present paper examines several of the major questions raised in this introduction. First, it remains a fact that no one has yet isolated an enzyme from any cartilage source and shown it to be cathepsin D. Moreover, the cathepsin D preparations used by the Strangeways group have not been rigorously shown to be cathepsin D as defined by Press et al. (18) on the basis of specificity of action on certain peptide bonds in the B chain of insulin. I set out to establish the presence of cathepsin D in the two distinct types of cartilage known to respond to vitamin A treatment: elastic cartilage from the ears of young rabbits and hyaline cartilage from the legs of chick embryos. A partial purification of cathepsin D from these two cartilage sources was reported in 1967 (17) along with data establishing the specificity of the enzymes. Interim reports (14, 19-22) have noted further progress leading to the present paper which reports considerable purification of both enzymes and describes completely their action on the B chain of insulin.

Secondly, the question of the pH range of cathepsin D action on protein-polysaccharide complexes can now be explored using both the newly available cartilage enzymes as well as a number of multiple forms of cathepsin D from bovine uterus (23). If cathepsin D can digest protein-polysaccharide complexes at neutral pH as previously suggested (15, 21), then the digesting activities at pH 5 and at pH 7.2 should bear some constant relationship to one another regardless of degree of purity, difference in species of origin, or multiplicity of forms. In fact, the results failed to show such relationships and ultimately led to the conclusion that cathepsin D has no action at pH values greater than 5.5 to 6.0.

**Experimental Procedure**

**Preparation of Cathepsin D from Rabbit and Chick Cartilage**—The assay of cathepsin D activity, the measurement of protein, and the definition of an enzyme unit have been described previously (24). The preparation of cathepsin D from rabbit ear cartilage is patterned closely on the methods used for bovine uterus (24). Carefully cleaned ear cartilage from young rabbits was obtained in frozen state from Pelfreeze Biologicals, Rogers, Ark. All steps were carried out in the cold. The cartilage from 90 ears was homogenized in 5 volumes of 0.5 M NaCl for 6 min in the VirTis homogenizer (VirTis, Inc., Gardiner, N. Y.). This provided about 1 liter of homogenate which was then purified by the same sequence of steps used for the cow uterus cathepsin D (24).

Preliminary studies indicate that 12- to 13-day-old chick embryos provided leg bones of optimal size and enzyme content. Leg bones at 7 to 8 days were too small to provide any reasonable quantity of enzyme; older bones began to lose their cartilaginous property and to develop calcium deposits in the midregion. Kimber- strain White Leghorn embryos were grown at 36.5°. At 12 to 13 days, the femurs and tibia were dissected out and collected in cold 0.9% NaCl solution. About 12 embryos were required to provide 1 g of cartilage. Eight grams of cartilage were homogenized for 2 min in 40 ml of distilled water in the VirTis homogenizer. The homogenate was centrifuged at 15,000 × g for 10 min. Solid NaCl was added to the supernatant to a final concentration of 0.5 M and the supernatant was placed on a column (2 × 9 cm) Sephadex G-100 equilibrated with 0.5 M NaCl. The enzyme was eluted with 0.5 M NaCl, and the active fractions were pooled and stored in the freezer. When 8 to 10 batches had been brought to this stage, they were all combined for the succeeding steps. The pH was adjusted to 3.5 with 1 N HCl (rapid stirring is critical here) and the material was incubated for 4 hours at 37°. The pH was then restored to 7.0 by the addition of 1 N NaOH. A slight precipitate was removed by a brief centrifugation. Solid (NH4)2SO4 was added to 40% saturation. The resulting precipitate was removed by centrifugation for 10 min at 15,000 × g. A second addition of (NH4)2SO4 brought the precipitate to 90% saturation. This second precipitate was then carried through Sephadex G-100 and DEAE-Sephadex in the same manner as the rabbit enzyme.

**Properties of Enzymes**—The pH activity curves for crude and purified preparations of both types of cartilage were based on hemoglobin digestion. The hemoglobin assay has been described earlier (24). The pH range from 1 to 6 was covered with acid-denatured hemoglobin in sodium citrate buffers; hemoglobin also contributed to the buffering capacity of the mixture. Above pH 5.5, urea-denatured hemoglobin and sodium phosphate buffers were used. Overlap was provided at pH 5.5 and 6.0. Final concentrations were: hemoglobin 1.2%, citrate 0.04 M, phosphate 0.04 M, urea 3.6 M, cysteine 0.025 M. Digestion was for 30 min at 37°.

The specificity of the two cathepsin preparations was tested on the S-sulfo B chain of insulin. Details of the B chain preparation and digestion and the separation and identification of the resultant peptides have all been described previously for the cow uterus enzyme (24). The very small amounts of cartilage cathepsins that could be obtained restricted the study of specificity. Digestions were done with 1 unit of enzyme and 1 mg of B chain, and the B chain products were fingerprinted. The patterns were almost identical with that obtained for cow uterus cathepsin D.

The conclusions as to specificity were based on a comparison of these patterns.

Molecular weight studies were based on movement of enzyme through Sephadex G-100 columns relative to the movement of ovalbumin (five times recrystallized, Penutex, Inc., Kankakee, Ill.).

**Preparation of Cathepsin D from Bovine Uterus**—The enzyme from bovine uterus was purified as described earlier (23). This enzyme occurs in multiple forms; the forms were partially separated by DEAE-cellulose chromatography (23). The final purification of these bands was accomplished by isoelectric focusing (25) using Ampholine, pH 5 to 8, in a 400-ml electrofocusing column (LKB-Produkter, Bromma, Sweden). The two major bands, forms 4 and 5, were also purified from the DEAE-cellulose peako by preparative disc electrophoresis using standard methodo (26) in a 2-cm long gel column in the Poly-Prep apparatus (Buehler Instruments, Fort Lee, N. J.). These forms were homogeneous by disc electrophoresis and had a specific activity of 140 units per mg of protein. The activity of all cathepsin preparations was assayed by hemoglobin digestion at pH 3.2 (24); 1 unit corresponds to the release of 6.6 μg of tyrosine per hour.
Preparation of Protein-Polysaccharide Complex—Bovine nasal cartilage was obtained fresh from the slaughter house, chilled immediately after excising, and then frozen. Protein-polysaccharide complex was extracted and purified by the method of Pal et al. (27) through their Step 1. This material is commonly designated protein-polysaccharide-light fraction or PP-L. An ether-dried powder of protein-polysaccharide complex was stored in a desiccator in the refrigerator. The dried protein-polysaccharide complex was swollen in 0.15 M KCl overnight and centrifuged at 105,000 × g for 1 hour to remove aggregated or insoluble material. The final concentration was about 10 mg of protein-polysaccharide complex per ml and this was then diluted and buffered for digestion experiments. It was usually found that protein-polysaccharide complex preparations incubated at pH 7.2 showed some endogenous proteolytic enzyme activity. This could be destroyed by brief exposure to urea. The dry protein-polysaccharide complex was dissolved in 8 M urea in the cold for 30 min, dialyzed exhaustively against 0.15 M KCl to remove the urea, and centrifuged as above. The final solution had essentially the same viscosity as protein-polysaccharide complex which had not been treated with urea and it lost all endogenous proteolytic activity.

Digestion of Protein-Polysaccharide Complex—The protein-polysaccharide complex was adjusted to the desired pH with HCl or NaOH and diluted with 0.15 M KCl to give a viscosity about 3 to 4 times that of water. This required a final concentration of about 5 mg per ml at pH values from 5 to 7. Most of the experiments were done without buffer addition, but with a pH check in the middle and at the end of digestion. However, for the pH curves and other indicated experiments, buffer was used at pH 6 and below, and sodium phosphate, at pH 7 and above.

Five milliliters of protein-polysaccharide complex solution were placed in a Cannon-Fenske viscometer and equilibrated in a controlled temperature viscometer bath (Neslab, Portsmouth, N. H.). Cathepsin D preparation (1 ml) was added and the flow time was measured with a stopwatch at 3- to 4-min intervals. The viscometers were calibrated with 0.15 M KCl and all viscosities were calculated relative to this standard. The endpoint of digestion was determined by digesting the protein-polysaccharide complex overnight. It was typically found that protein-polysaccharide complex with an initial ηrel of 4.0 gave a final ηrel of 1.75.

**RESULTS**

Purification of Cathepsin D from Rabbit Ear Cartilage—This purification proceeds in much the same way as the purification of cathepsin D from bovine uterus. Only minor modifications need be introduced. Some difficulty is experienced in dispersing the cartilage; vigorous homogenization with high shearing is required. The resulting homogenate does not pack well in the centrifuge; about 25% of the fluid and enzyme units remain in the pellet. The progress of the purification is detailed in Table I, which presents the average of three preparations. The final yield and specific activity are comparable to the values obtained for cow uterus cathepsin D (24).

Purification of Cathepsin D from Chick Embryo Limb Cartilage—The embryo cartilage presented a difficult problem in purification. The major problem was the high viscosity of the extracts. Saline extracts did not pack as well as aqueous extracts upon centrifugation. Then, if one attempted the usual acid dialysis step, a heavy precipitate formed which entrapped all of the enzyme activity. The final scheme of purification is summarized in Table II (average of three preparations). Again, the yield and purification factor are similar to those obtained with cow uterus and rabbit ear sources. However, the specific activity of the chick enzyme is double that of the rabbit ear enzyme and equals that of highly purified cow uterus cathepsin D (23). Disc electrophoresis indicates that the chick enzyme is not completely pure at this stage; both it and the rabbit enzyme are estimated to be 40 to 50% pure. Hence, the chick enzyme is expected to show an ultimate specific activity double that of the cow and rabbit enzyme. This prediction is supported by the results of Barrett (28), who finds that the specific activity of cathepsin D from adult chicken liver is almost double that for the human liver enzyme.

Properties of Two Cathepsin Preparations—Fig. 1 shows the pH activity curves for hemoglobin digestion by the crude and purified cartilage enzymes. Between pH 2 and 6 the pH profile is essentially identical for crude and 900-fold purified enzymes. This is interpreted to mean that all of the hemoglobin-digesting capacity below pH 5 in the crude homogenates is accounted for by cathepsin D. There is some evidence for the presence of cathepsin B in the rabbit ear cartilage, as shown by the cysteine activation at pH 5 to 6. This activity disappears upon purification. The chick enzyme shows no evidence of a sulfhydryl-activated cathepsin. Both homogenates show slight activity at pH 7.5, but this, too, is lost upon purification. The chick and rabbit enzymes both show the same pH optimum at pH 3.0 to 3.2. The rabbit enzyme shows a shoulder at pH 4.5; a similar shoulder has also been found for the cow uterus enzyme (24). This shoulder is typical for cathepsin D and does not represent another enzyme activity. The chick enzyme has a less pronounced shoulder.

The identification of cathepsin D must ultimately be based on its specificity toward the B chain of insulin as described by Press et al. (27) through their Step 1. This material is commonly designated protein-polysaccharide-light fraction or PP-L. An ether-dried powder of protein-polysaccharide complex was stored in a desiccator in the refrigerator. The dried protein-polysaccharide complex was swollen in 0.15 M KCl overnight and centrifuged at 105,000 × g for 1 hour to remove aggregated or insoluble material. The final concentration was about 10 mg of protein-polysaccharide complex per ml and this was then diluted and buffered for digestion experiments. It was usually found that protein-polysaccharide complex preparations incubated at pH 7.2 showed some endogenous proteolytic enzyme activity. This could be destroyed by brief exposure to urea. The dry protein-polysaccharide complex was dissolved in 8 M urea in the cold for 30 min, dialyzed exhaustively against 0.15 M KCl to remove the urea, and centrifuged as above. The final solution had essentially the same viscosity as protein-polysaccharide complex which had not been treated with urea and it lost all endogenous proteolytic activity.

Digestion of Protein-Polysaccharide Complex—The protein-polysaccharide complex was adjusted to the desired pH with HCl or NaOH and diluted with 0.15 M KCl to give a viscosity about 3 to 4 times that of water. This required a final concentration of about 5 mg per ml at pH values from 5 to 7. Most of the experiments were done without buffer addition, but with a pH check in the middle and at the end of digestion. However, for the pH curves and other indicated experiments, buffer was used to give a final concentration of 0.05 M. Sodium acetate buffer was used at pH 6 and below, and sodium phosphate, at pH 7 and above.

Five milliliters of protein-polysaccharide complex solution were placed in a Cannon-Fenske viscometer and equilibrated in a controlled temperature viscometer bath (Neslab, Portsmouth, N. H.). Cathepsin D preparation (1 ml) was added and the flow time was measured with a stopwatch at 3- to 4-min intervals. The viscometers were calibrated with 0.15 M KCl and all viscosities were calculated relative to this standard. The endpoint of digestion was determined by digesting the protein-polysaccharide complex overnight. It was typically found that protein-polysaccharide complex with an initial ηrel of 4.0 gave a final ηrel of 1.75.

**TABLE I**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Total units</th>
<th>Ratio of cathepsin D to protein</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>950</td>
<td>270</td>
<td>0.063</td>
<td>1</td>
</tr>
<tr>
<td>Citrate dialysis</td>
<td>860</td>
<td>185</td>
<td>0.173</td>
<td>2.7</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>60</td>
<td>150</td>
<td>0.173</td>
<td>2.7</td>
</tr>
<tr>
<td>Dioxane</td>
<td>3.2</td>
<td>141</td>
<td>2.53</td>
<td>40</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>48</td>
<td>125</td>
<td>21.5</td>
<td>340</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>28</td>
<td>106</td>
<td>56</td>
<td>890</td>
</tr>
</tbody>
</table>

**TABLE II**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Total units</th>
<th>Ratio of cathepsin D to protein</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude aqueous extract</td>
<td>350</td>
<td>262</td>
<td>0.156</td>
<td>1</td>
</tr>
<tr>
<td>First Sephadex G-100</td>
<td>355</td>
<td>233</td>
<td>0.70</td>
<td>4.4</td>
</tr>
<tr>
<td>Autolysis; (NH₄)₂SO₄</td>
<td>10.5</td>
<td>214</td>
<td>2.08</td>
<td>12.6</td>
</tr>
<tr>
<td>Second Sephadex G-100</td>
<td>43</td>
<td>159</td>
<td>15.4</td>
<td>99</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>12</td>
<td>120</td>
<td>141</td>
<td>906</td>
</tr>
</tbody>
</table>
Fig. 1. A, pH activity curve for the digestion of denatured hemoglobin by cathepsin D from rabbit ear cartilage. , crude homogenate; , purified cathepsin; X, crude homogenate plus cysteine. B, pH activity curve for the digestion of denatured hemoglobin by cathepsin D from chick limb cartilage. No cysteine effects could be detected. Details are given under “Experimental Procedure.”

e et al. (18). Fig. 2 shows a comparison of B chain fingerprints for the cartilage enzymes and the cow uterus enzyme. The eight major peptide and amino acid spots are identical in every case. It was previously shown that these eight strong spots result from major points of cleavage at Leu₁₅-Tyr₁₆, Tyr₁₆-Leu₁₇, Phe₂₀-Phe₂₁, and Phe₂₀-Tyr₁₆ and secondary points of cleavage at Phe₁₁-Val₁₀, Glu₁₁-Ala₁₀, Ala₁₁-Leu₁₁, and Tyr₁₆-Leu₁₁. This specificity is essentially that reported by Press et al. (18) except for the additional weak splitting at Phe₁₁-Val₁₀ and Ala₁₁-Leu₁₁. The present study also shows some splitting at Tyr₁₆-Thr₁₇ which was not found in earlier studies of the uterus enzyme (24). All these minor splits have been observed in other species of cathepsin D (24).

Two faint spots in Fig. 2 (a, b), seen in all three patterns, have been traced to impurities in the B chain preparation. Spot c, seen only in the cow and rabbit patterns, is identified as Thr₁₇-Ala₃₉. This arises from the peptide immediately to the left by the cleavage of the Tyr₁₆-Tyr₁₇ bond. The chick embryo enzyme shows several spots that are intermediates in the digestion pathway. Spot d is Tyr₁₆-Phe₂₀; in the other patterns this peptide has lost its Tyr₁₆ and lies to the left. Spot e is Phe₂₀-Ala₂₀; the loss of Phe₂₀ produces the peptide immediately below it. The very faint spot f has not been identified. The chick enzyme, therefore, does not differ importantly from the other two. All but one difference can be attributed to a slightly slower action of the enzyme so that several peptides remain incompletely digested.

Both enzymes emerge from Sephadex G-100 columns in the same volume relative to the void volume (V₀/Vᵥ = 0.58), and this is found to be exactly the same volume as for ovalbumin and uterine cathepsin D. Therefore, the weight is presumed to be 40,000 to 43,000 (23, 29). The molecular weight of the chick enzyme was not altered by the autolysis step.

Action of Cathepsin D on Protein-Polysaccharide Complex at pH 5—The first experiments were done with the most readily available and most highly purified preparation of cathepsin D, form 4 from the bovine uterus. When 1 unit of cathepsin D was added to 5 ml of protein-polysaccharide complex solution in a viscometer at pH 5.0 and 25°C, there was a rapid drop in the viscosity of the protein-polysaccharide complex. Based on the endpoint calculated from the minimum viscosity reached after 18 hours, it was found that about 85% of the over-all viscosity change occurred in the 1st hour. Several methods of analyzing the data were tried and the most useful was found to be a second order plot based on the viscosity change that occurred by any time t divided by the viscosity change still to occur from time t until digestion was complete. Fig. 3 shows such a plot for several different concentrations of enzyme. It can be seen that the plot is linear over about 85% of the reaction course, the ordinate value of 4 corresponds to 85.7% of the viscosity change completed divided by 14.3% remaining to be completed. It will be further noted that the assay is linear with respect to enzyme concentration; 0.5 unit of enzyme gives a slope (second order rate constant) exactly 1/4 of that given by 1 unit, etc. This means that the viscosimetric method at pH 5 is suitable for use in enzyme assays and in testing the effect of inhibitors. In the absence of added buffers, linearity of the second order plots is not obtained at lower pH values. This was one reason for doing most of the studies at pH 5, even though the pH optimum is close to
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Fig. 3. Viscosimetric measurements of the digestion of protein-polysaccharide complex by cathepsin D from bovine uterus in 0.15 M KCl and 0.05 M sodium acetate pH 5.0 and 25°. Details are given under “Experimental Procedure.” The flow time of the mixture relative to that of 0.15 M KCl was measured at various time intervals. The relative viscosity of the mixtures was found to decline at a second order rate; that is, the viscosity change from 0 to time t divided by the viscosity change from time t to complete digestion was a linear function of time.

pH 4. The other reason was to be closer to physiological pH and to the pH used by others who had studied matrix digestion.

The assay has a number of elements of variability in it. Different protein-polysaccharide complex preparations give different second order rate constants with a fixed level of cathepsin, the rate is sensitive to pH changes, and there are also uncertainties as great as 10% in the assay of cathepsin D (23). Therefore, the most meaningful results are those which were obtained on the same day using a given preparation of protein-polysaccharide complex and which compare various enzymes or inhibitors under the same set of conditions.

The digestion products were examined by several physical techniques. Fig. 4 shows the results of electrophoresis and chromatography. The protein-polysaccharide complex preparation has a high molecular weight and remains at the origin in both cases. Purified chondroitin sulfate is relatively free of protein and shows the greatest migration. The products of cathepsin D are intermediate between these two extremes, showing degradation, but not to single chains of chondroitin sulfate. It is possible that the products are doublet chains of polysaccharide as suggested by the studies of Luscombe and Phelps (6). Fig. 5 shows the schlieren pattern in the analytical ultracentrifuge. The protein-polysaccharide complex preparations are not homogeneous but they do give a single broad peak. The digestion products also give a single broad peak, but the sedimentation coefficient is considerably smaller. The higher weight material completely disappears on digestion and gives rise to smaller species, none of which was present initially. The undigested protein-polysaccharide complex had an $s_{20,w}$ of 6.8 at a concentration of 5 mg per ml. The digestion products had an $s_{20,w}$ of 2.6. There can be no doubt that the original molecules of protein-polysaccharide complex are completely degraded to smaller fragments.

Inhibition of Protein-Polysaccharide Complex Digestion—Fig. 6 shows the results of various inhibitors tested for their effect on the digestion of protein-polysaccharide complex at pH 5 by cathepsin D from bovine uterus. e-Aminocaproic acid and arginine are inhibitory at high concentrations (0.1 M). Chloroquine and 3-indolepyruvate inhibit the activity about 85% at a concentration of 20 mM. Pepstatin is a very powerful inhibitor,
already considerable ionic strength from the 0.15 M saccharide complex is almost exactly doubled by adding 0.05 M. It is found that the activity of cow cathepsin D on protein-polysaccharide complex mixtures. This has been studied in detail at pH 5, where the point of interest is that the buffer enhances the digestion of protein-polysaccharide complex compared with unbuffered incubation. When this was done it was found that second order curves could be obtained at almost every pH. It was also decided to compare all three preparations of cathepsin D to explore species differences.

Optimum pH for Protein-Polysaccharide Complex Digestion—In initial studies (17) protein-polysaccharide complex digestion was studied in unbuffered solutions at various pH's and the optimum digestion was found at about pH 4. The studies were difficult because the kinetic behavior was not second order below pH 5. It was decided to reinvestigate the pH curve using 50 mM buffers. The resultant pH curves are presented in Fig. 7. In each case the pH optimum occurs at pH 4, and the activity falls almost to 0 by pH 5.5. At the optimum pH 4.1 unit of rabbit enzyme has only 1/2 the activity on protein-polysaccharide complex shown by 1 unit of cow enzyme. The chick enzyme has the least activity on protein-polysaccharide complex; it has about 13% of the cow activity at pH 4 and 22% at pH 5. Another point of interest is that the buffer enhances the digestion of protein-polysaccharide complex compared with unbuffered incubation mixtures. This has been studied in detail at pH 5, where it is found that the activity of cow cathepsin D on protein-polysaccharide complex is almost exactly doubled by adding 0.05 M acetate buffer. The reason for this effect is not clear; there is already considerable ionic strength from the 0.15 M KCl and the pH did not change in the unbuffered samples.

The initial viscosity of protein-polysaccharide complex is quite sensitive to pH changes. As the pH is reduced below 5.0, the relative viscosity becomes lower. By pH 3.0 the relative viscosity of protein-polysaccharide complex has declined from 4.0 to 2.2. Also at pH 3 there is a rapid spontaneous change in viscosity. This may be due to an endogenous protease acting at a pH lower than cathepsin D. In any event, it was impossible to get meaningful data at pH 3 by viscometric means.

Action of Cathepsin D on Protein-Polysaccharide Complex at pH 7.2—Earlier preparations of cow cathepsin D were found to digest protein-polysaccharide complex slowly at pH 7 (17). This digestion was magnified about 12-fold by using 3 units of cathepsin and raising the temperature to 37°. More recently we have achieved purities of cathepsin D about double those used in the original experiments (23). Moreover, several multiple forms of the uterus enzyme were available in addition to the two cartilage species of enzyme. This suggested a test of the hypothesis that cathepsin D action on protein-polysaccharide complex at pH 7.2 might be due to a contaminating protease. The test would be that the action of the different enzyme preparations on protein-polysaccharide complex at pH 7.2 should be in the same proportion to their hemoglobin-digesting activity as their action on protein-polysaccharide complex at pH 5. If such proportionality were found it would argue against the hypothesis.

The first test involved the five most common multiple forms of uterine cathepsin D as well as the two cartilage enzymes. The results, shown in Fig. 8, were found to be different for each enzyme preparation. Each multiple form gave a different activity at pH 7.2, whereas at pH 5.0, they all digested protein-polysaccharide complex equally. The different activities bear no relation to the charge distribution on the enzyme, so they do not appear to be due to different binding affinities of enzyme for substrate. Most interesting was the observation that the chick enzyme had no detectable action on protein-polysaccharide complex at pH 7.2. These findings prompted further investiga-
lost all its pH 7.2 activity when stored in the refrigerator. Al-
doubled to 14.2 units/3 units of D. Band 5 of preparation 102
unit of this enzyme was tested at pH 7.2 its activity was now
high activity of about 7 units at pH 7.2. After freezing for 6
loss is not always reproducible. Uterus preparation 86 had a
zyme which was lost on storage in the freezer. However, this
at pH 7.2. This indicated the presence of a contaminating en-
tron protein-polysaccharide complex it was found to have no effect
and the other was frozen. When the frozen sample was tested

tations and certain inhibitors.

Table III presents a detailed comparison of different cathepsin
preparations at pH 5.0 and 7.2. All of the cow uterus prepar-
arations digested protein-polysaccharide complex at pH 5 to the
same extent when 1 hemoglobin unit was tested. Pepstatin
chloroquine inhibited strongly at pH 5 as noted earlier. Neither compound inhibited the digestion of protein-polyc-
saccharide complex at pH 7.2. In fact, increasing the pepstatin
concentration 100-fold still had no discernible effect at pH 7.2.
It was shown previously that pepstatin binds to cathepsin D
from the cartilage of these animals.

FIG. 8. Viscosimetric assay of protein-polysaccharide com-
plex digestion by various preparations of cathepsin D at pH 7.2,
37°C. Three units (hemoglobin assay at pH 3.2) of cathepsin D
were used in each case and the protein-polysaccharide complex
was buffered with 0.05 M sodium phosphate. FORMS 3 to 7 refer
to the distinct multiple forms of cathepsin D prepared from
bovine uterus (24). CHICK and RABBIT refer to cathepsin D
from the cartilage of these animals.

tion of the action at pH 7.2, using additional enzyme prepara-
tions and certain inhibitors.

Table III presents a detailed comparison of different cathepsin
preparations at pH 5.0 and 7.2. All of the cow uterus prepar-
arations digested protein-polysaccharide complex at pH 5 to the
same extent when 1 hemoglobin unit was tested. Pepstatin
chloroquine inhibited strongly at pH 5 as noted earlier. Neither compound inhibited the digestion of protein-polyc-
saccharide complex at pH 7.2. In fact, increasing the pepstatin
concentration 100-fold still had no discernible effect at pH 7.2.
It was shown previously that pepstatin binds to cathepsin D
from the cartilage of these animals.

When uterus preparation 102 was originally prepared it was
divided into two lots; one was stored in the refrigerator at pH 8
and the other was frozen. When the frozen sample was tested
on protein-polysaccharide complex it was found to have no effect
at pH 7.2. This indicated the presence of a contaminating en-
yze which was lost on storage in the freezer. However, this
loss is not always reproducible. Uterus preparation 86 had a
high activity of about 7 units at pH 7.2. After freezing for 6
months, ½ of the cathepsin D activity was lost. When 1 D
unit of this enzyme was tested at pH 7.2 its activity was now
doubled to 14.2 units/3 units of D. Band 5 of preparation 102
lost all its pH 7.2 activity when stored in the refrigerator. Al-
though the stability properties of the pH 7.2 activity are not
well understood, it is amply clear that this activity varies inde-
pendently of the cathepsin D activity.

Preparation 104 was purified by using preparative disc elec-

trophoresis in the final step. This enzyme showed no action
at pH 7.2; and it has since been possible to obtain several prepa-
arations of uterus cathepsin D which are free of activity at pH
7.2. The best preparation obtained to date had a specific ac-
tivity of 155 units per mg of protein; 20 units of this preparation
acting on protein-polysaccharide complex at pH 7.2 produced a
second order rate constant of less than 0.002 per hour (5% vis-
cosity change in 24 hours).

The rabbit preparations had about ½ the activity at pH 5
that the cow uterus enzyme had. The activity at pH 7.2 was
relatively high. The chick enzyme 42 had no activity at pH 7.2,
whereas enzyme 43 had a slight trace of activity. It is con-
cluded on the basis of the high variability or complete absence
of pH 7.2 activity, that this activity is not due to cathepsin D
but is due to a second, contaminating, protease (or proteases).
Further studies show that the cathepsin D action is barely per-
ceptible above pH 5.5; activity seen at pH 6 is directly propor-
tional to the activity at pH 7.2 and not to the activity at pH 5.

A number of the points established above with protein-polyc-
saccharide complex were also verified using the more uniform
proteoglycan subunit preparation of Hascall and Sajdera (4).
It was shown that certain cow enzyme preparations digested
proteoglycan subunit at pH 7.2 and that these were not inhibited
by pepstatin or diisopropyl fluorophosphate. It was also shown
that cow preparation 104 and chick preparation 42 had no action
on proteoglycan subunit at pH 7.2.

**DISCUSSION**

In the present study it is shown unambiguously by purifica-
tion and specificity studies that cathepsin D is the major acid

cathepsin (based on hemoglobin digestion) in the hyaline car-
tilage of chick embryo limbs and the elastic cartilage of rabbit
cats. The development of purification methods giving reasona-
ably high recoveries is of critical importance when one
considers the difficulties of obtaining the source material. Each
batch of enzyme in the present study required the ears of 45

<table>
<thead>
<tr>
<th>Enzyme incubation</th>
<th>pH 5.0, 25°C, 1 unit cathepsin D</th>
<th>pH 7.2, 37°C, 3 units cathepsin D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow 102, form 4, refrigerated 4 months</td>
<td>5.6 ± 0.2</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>Cow 102, form 4 + pepstatina</td>
<td>0.17 ± 0.01</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>Cow 102, form 4 + chloroquine, 20 mM</td>
<td>0.64 ± 0.02</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Cow 102, form 4, frozen 4 months</td>
<td>5.6 ± 0.2</td>
<td>0 ± 0.1</td>
</tr>
<tr>
<td>Cow 86, form 5, frozen 6 monthsb</td>
<td>5.5 ± 0.1</td>
<td>14.2 ± 0.2</td>
</tr>
<tr>
<td>Cow 104, form 4, fresh</td>
<td>5.7 ± 0.2</td>
<td>0 ± 0.1</td>
</tr>
<tr>
<td>Cow 102, form 5, refrigerated 4 months</td>
<td>5.6 ± 0.2</td>
<td>0 ± 0.1</td>
</tr>
<tr>
<td>Chick 42, frozen 1 year</td>
<td>1.05 ± 0.01</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>Chick 43, fresh</td>
<td>1.2 ± 0.1</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>Rabbit 7</td>
<td>2.8 ± 0.1</td>
<td>4.3 ± 0.1</td>
</tr>
</tbody>
</table>

* Activity measured in second order rate units per hour as in
  Fig. 1.

a Concentrations were 2.5 x 10⁻³ M at pH 5 and 2.5 x 10⁻⁴ M
  at pH 7.2.

This preparation originally had 6.3 units of activity at pH 7.2.
After freezing, more than half of the cathepsin D activity was
lost. When 3 hemoglobin units were tested, they produced
protein-polysaccharide complex digestion of 14.2 units, indicating
that none of the protein-polysaccharide complex digestion activity
had been lost.
on the basis of pH optimum (12) or have demonstrated cathepsin function was found to be useful over a considerable pH range, nor by the function l/a" used by Almin and Ericksson (30) and Hulme (31) to describe the enzymic digestion of carboxymethylcellulose. On the other hand, the second order function was found to be useful over a considerable pH range and with a wide range of enzyme concentrations.

The digestion of protein-polysaccharide complex at pH 4 to 5 by cathepsin D suggests that this enzyme may account for some previously observed examples of cartilage enzymes which attacked the matrix at pH 4.5. The activities described by Dziewiatkowski (13) and Ali (11) may well have been due to cathepsin D, at least in part. It should be noted that the present paper is the first to demonstrate the breakdown of a matrix component by a cartilage protease unequivocally identified as cathepsin D. Other studies have assumed cathepsin D action on the basis of pH optimum (12) or have demonstrated cathepsin D indirectly by immunological methods (15).

It might be asked whether the action of cathepsin D at pH 5 is truly due to this enzyme or to some proteolytic impurity. Particular concern is raised by the action of certain inhibitors which would seem to be specific for a trypsin-like enzyme rather than a cathepsin-like enzyme. Both L-aminocaproic acid and arginine might be expected to inhibit because of their similarity to trypsin substrates. These compounds do not inhibit the action of cathepsin D on hemoglobin at pH 3.2. On the other hand, the cathepsin D preparations have no action on benzoylarginine amide (24). Moreover, there is no cathepsin B action, as indicated by the failure of iodoacetate to inhibit. It is concluded that L-aminocaproic acid and arginine inhibit protein-polysaccharide complex digestion, not because there is a cathepsin B-type activity in the preparations (11), but because of physical interaction with the high negative charge on the substrate molecules. This interpretation is supported by the high inhibitor concentrations (0.1 M) required to produce inhibition. Barrett's (32) report that 3-indolepyruvic acid inhibits cathepsin D was confirmed earlier (24) and this compound is also shown here to block protein-polysaccharide complex digestion. Chloroquine phosphate was shown by Cowey and Whitehouse (33) to block the degradation of matrix in cartilage slices incubated at pH 5. The present results suggested this effect might be due to an action on cathepsin D. Again, this compound has no action on hemoglobin digestion by the enzyme.

The most telling piece of evidence for cathepsin D being the responsible enzyme is the almost total inhibition by pepstatin.

This compound is a very potent inhibitor of pepsin and pepsin-like enzymes (34). It inhibited protein-polysaccharide complex digestion 97% at a concentration of 2 x 10⁻² M. Another piece of evidence is that protein-polysaccharide complex-digesting activity increased pari passu with hemoglobin digesting activity as cathepsin D was purified from 400-fold to 2000-fold. It is concluded, therefore, that essentially all of the digestion of protein-polysaccharide complex at pH 5 is due to the action of cathepsin D and that the ability of certain compounds to inhibit protein-polysaccharide complex breakdown but not hemoglobin digestion is related to the different properties of the two substrates.

The most important conclusion reached in the present paper is that cathepsin D has no detectable action on protein-polysaccharide complex or proteoglycan subunit at physiological pH. Because of earlier, contradictory reports from my laboratory (17) and others (14, 15), it is important to consider the evidence for this conclusion quite carefully. The most important piece of evidence is, of course, the eventual preparation of cow uterus and chick cartilage catecholamines which were completely incapable of reducing the viscosity of protein-polysaccharide complex or proteoglycan subunit solutions at pH 7.2 even when the enzyme level was increased to 20 units and incubation was extended to 24 hours. The earlier observations presumably had their origin in contaminating proteases (or proteases) with neutral pH optimum. This is supported by the finding that most of the cathepsin D preparations examined (about 20 in all) showed protein-polysaccharide complex-digesting action at pH 7.2. However, this activity varied widely with respect to the hemoglobin-digesting activity. It does not parallel the purification of cathepsin D; it did not parallel losses of cathepsin D on storage, and it was not inhibited by inhibitors of cathepsin D. It must be concluded that cathepsin D is unlikely to have any significant effect on the cartilage matrix at pH values greater than 5.5 to 6.0.

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