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CATHEPSINS B1 AND D

ACTION ON HUMAN CARTILAGE PROTEOGLYCANS

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SUMMARY

1. Because of the possibility that the lysosomal proteinases, cathepsins B1 and D, play a part in both normal and pathological catabolism of the extracellular matrix of human articular cartilage, we have examined the action of the pure enzymes on human cartilage and its component proteoglycans.

2. Human cathepsins B1 and D were shown by viscometry to degrade proteoglycans from adult human articular cartilage in solution. Gel chromatography of the products on Sepharose 4B indicated that those produced by cathepsin B1 were similar to those resulting from the action of papain, which contain a single polysaccharide chain. Cathepsin D produced fragments similar to those resulting from the action of trypsin, which contain two polysaccharide chains.

3. Both cathepsins B1 and D released proteoglycans from ^{35}S-labelled human embryonic cartilage that had been washed free of endogenous lysosomal enzymes. The release was most rapid at pH 5.0 with both enzymes.

The autolytic release of proteoglycans from cartilage incubated in various buffers was also most rapid at pH 5.0.

4. When autolysis or treatment of washed cartilage with purified cathepsin D was carried out in the presence of sheep anti-(human cathepsin D) serum, up to 70% inhibition of the release of labelled proteoglycan occurred.

5. It is concluded that the results are consistent with both cathepsins B1 and D playing a part in the catabolism of cartilage matrix.

INTRODUCTION

Previous work from this laboratory has provided evidence that under certain conditions cathepsin D plays a major role in the breakdown of cartilage matrix of various species[1,2]. Using immunochemical methods, Dingle and coworkers[2] have shown that cathepsin D is mainly responsible for the release of proteoglycan from chick or rabbit ear cartilage during autolytic breakdown (see also Morrison[3,4] and

Weston *et al.*[5]). Woessner[6] studied the proteolytic activities of rabbit ear and chick limb bone cartilage and found that they were of the cathepsin D type, and were sufficient quantitatively to account for the degradation of the matrix. Ali and Evans[7] investigated the autolytic breakdown of rabbit ear cartilage and reported that although cathepsins A, B, C and D were all present, cathepsin D was mainly responsible for the degradation. Earlier suggestions that cathepsin B played a major role in the autolytic breakdown of this tissue (Ali[8]; Ali *et al.*[9]) now appear to be excluded (Dingle *et al.*[2]). Recent work using pepstatin, an inhibitor of carboxyl-dependent acid proteinases (Dingle *et al.*[10]), has demonstrated a major role for cathepsin D in the autolytic degradation of adult human articular cartilage and a lesser, but significant effect of a thiol-dependent enzyme. Dingle[11] suggested that lysosomal acid proteinases may play an important role in the degradation of human cartilage in arthritis, whereas other workers[12,13] have proposed that other enzymes may also be involved in the disease process. Since much previous work has been concerned with species other than man, the present experiments were undertaken further to elucidate the enzymic mechanism of the degradation of human cartilage and the way in which purified enzymes can attack proteoglycans isolated from this tissue. We shall present evidence consistent with the possibility that both cathepsins D and B1 may be concerned in this process.

MATERIALS

Carrier-free sodium [^{35}S]sulphate in aqueous solution was obtained from the Radiochemical Centre, Amersham, Bucks, U.K. Sepharose and Sephadex for gel chromatography were obtained from Pharmacia (G.B.) Ltd, London W.13. Twice-crystallised papain and trypsin were bought from Sigma London Chemical Co., Surrey, KT2 7BH. Cathepsin D (EC 3.4.4.23) was purified from human liver essentially as described by Barrett[14] and cathepsin B1 (in the terminology of Barrett and Dingle[15]) from this tissue was prepared by the method of Barrett[16]. The enzyme was shown to be homogeneous on isoelectric focusing on polyacrylamide gel and gave rise to a monospecific antiserum when used to immunize sheep[16]. Several separate preparations had the same amino acid composition (Barrett, A. J., unpublished work). All reagents were of Analar grade, when this was available.

METHODS

Raising and testing of antisera, and experiments involving autolysis of cartilage, were as described by Dingle *et al.*[2].

Labelling of human cartilage

The foetuses were obtained from patients referred for termination of pregnancy under Sections 2 and 3 of the Abortion Act 1967. Between 15 and 18 weeks gestation each foetal sack was removed *in toto* after anterior hysterotomy. It was immediately transferred to a plastic container and placed on ice until it reached the laboratory. Within 1 h the epiphyses of the long bones of the foetus were dissected free of soft connective tissue, sliced and incubated at 37 °C in BGJ_5 medium[17] free of unlabelled sulphate, but containing 100 μCi [^{35}S]sulphate/ml. The tissue was incubated in a

roller bottle apparatus for 24 h in a fluid to tissue ratio of 2.5:1 (w/v). Typically, 2 μCi ^{35}S were incorporated into ethanol-precipitable proteoglycan/g tissue.

Removal of endogenous enzymes from cartilage

Tissue was twice frozen and thawed, and extracted for 2 h at 0 °C in 100 vol. (v/w) of 0.1 M NaCl containing 0.1% Triton X-100 and 0.02 M phosphate buffer, pH 7.0. The tissue was further extracted for 24 h in a fresh portion of the same solution. Part of the cartilage was homogenised and assayed for cathepsin D. The enzyme was not detectable, *i.e.* less than 5% of the original activity of the tissue remained.

Preparation of proteoglycans from human cartilage

Proteoglycan was a mixture of protein–polysaccharide complexes isolated by the dissociative extraction method of Sajdera and Hascall[18]. Normal adult human cartilage obtained *post mortem* from the femoral head was diced and extracted for 24 h in 10 vol. of 4 M guanidinium chloride in 0.05 M sodium acetate buffer, pH 5.8, with stirring. The cartilage residues were removed by filtration, and the proteoglycans were precipitated from the filtrate with 4 vol of ethanol containing 2% (w/v) potassium acetate. The precipitate was twice redissolved in water and reprecipitated in the same way. For some experiments, light proteinpolysaccharide preparations were made as described by Gerber *et al.*[19].

Scintillation counting

Aqueous samples were counted in a Packard Tricarb Liquid Scintillation Spectrometer Model 3375 in 10 ml of scintillant solution[20] of composition appropriate to the volume of aqueous sample.

Gel chromatography

Column chromatography was carried out in tubes of dimensions 1.5 cm × 30 cm (Pharmacia, Type K15/30) containing 40–50 ml columns of Sephadex or Sepharose. Columns were packed as recommended by the manufacturers and elution was with 0.15 M NaCl containing 0.05 M sodium acetate buffer, pH 5.5, and 0.03% toluene as antibacterial agent. Flow rates not exceeding 6 ml/cm^2 per h were obtained by use of a peristaltic pump (Watson Marlow Ltd, Falmouth, Cornwall, U.K., Type D/K6M). Fractions were analysed for uronic acid by the method of Bitter and Muir[21], for radioactivity by liquid scintillation counting or for anionic polysaccharide by the method of DiFerrante[22], chondroitin sulphate being used as standard[2].

Viscometry

Viscometric determinations were carried out in miniature Ostwald viscometers having a water flow time of about 30 s (A. Gallenkamp, Type UM-4). Flow times were measured with an electronic timer. Solutions contained *n*-butanol (1%) or toluene (0.03%) as preservative during long incubations.

Units of enzyme activity

The units of activity of human cathepsins B1 and D were as defined by Barrett (refs 23 and 14, respectively), except that 1 unit of cathepsin B1 released 1 μmole of

naphthylamine from benzoyl-D,L-arginine 2-naphthylamide/min, rather than 1 nmole. The molecular weights have been taken as 27 000 [16] and 43 000 [24] and the specific activities (on the basis of protein determined by the method of Lowry *et al.*[25] with bovine serum albumin as standard) 5.5 [16] and 600 [14]. From these data it was calculated that 1 unit of cathepsin B1 represents 6.7 nmoles and of cathepsin D, 0.038 nmole. Where it has been important to compare the activity of the two enzymes, their concentrations have been expressed in terms of nmoles/ml.

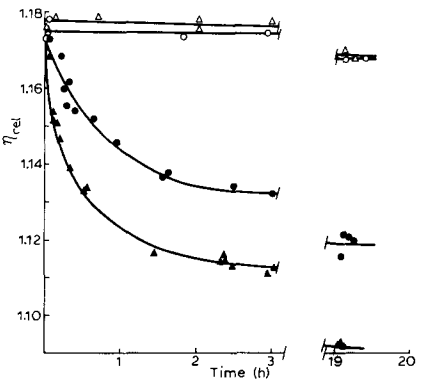
RESULTS

Action of purified enzymes on proteoglycan in solution

When proteoglycan from adult human articular cartilage was treated with cathepsin B1 or cathepsin D, the viscosity of the solution was rapidly reduced (Fig. 1). The final viscosity was lower with cathepsin B1 than with cathepsin D.

After incubation in the viscometer, the proteoglycan solutions were subjected to gel chromatography on Sepharose 4B. The reduced molecular size of the degraded molecules as compared with the undegraded material was seen as greater retardation on the column. Further treatment of the digestion products with fresh cathepsin D gave fragments appearing at the same percentage of the column bed volume as those produced by trypsin; no material was then excluded from the gel (Fig. 2).

After overnight incubation, the proteoglycan treated with cathepsin B1 contained a considerable amount of material of a lower molecular weight distribution



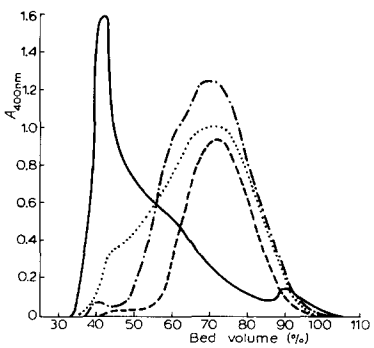


Fig. 1. Effect of human cathepsins B1 and D on viscosity of proteoglycan from human articular cartilage. The final concentration of proteoglycan was 2.53 mg/ml. Treatment with cathepsin D (0.18 nmole/ml) was carried out in 0.15 M NaCl containing 0.05 M sodium acetate buffer, pH 5.0 (●). Treatment with cathepsin B1 (0.66 nmole/ml) was carried out in the same solvent system, but with the addition of 1 mM EDTA and 2 mM cysteine (▲). Control incubations were carried out in the appropriate buffer systems without the enzymes (○ and △, respectively). The experiments were made at 37 °C.

Fig. 2. Gel chromotography on Sepharose 4B of proteoglycan and degradation products resulting from the action of cathepsin D and trypsin. Elution profiles of undegraded material (———) and of products after overnight incubation with cathepsin D under the conditions described in Fig. 1 (— · —), after a further overnight incubation with 0.30 nmole fresh cathepsin D/ml (— — —). The digestion of proteoglycan (2.66 mg/ml) with trypsin (5 nmole/ml) was in 0.02 M Tris–HCl buffer, pH 7.9, at 37 °C for 16 h (· · · · · ·). The products were detected by the turbidimetric method[2].

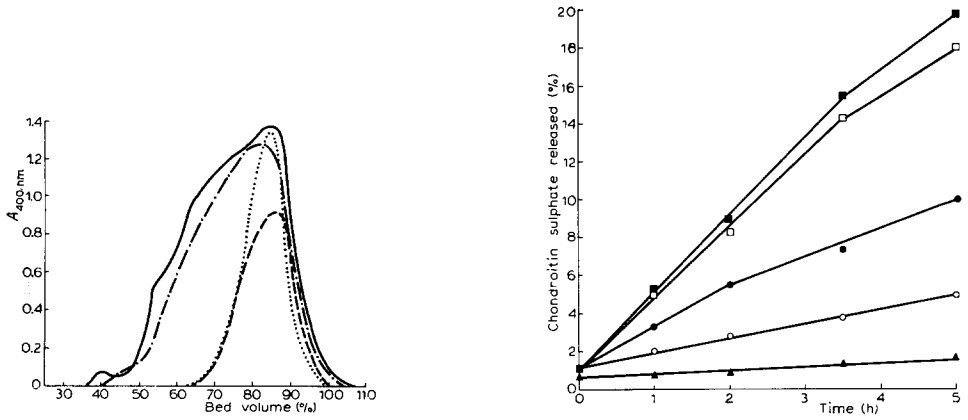


Fig. 3. Gel chromatography on Sepharose 4B of proteoglycan and degradation products resulting from the action of cathepsin B1 and papain. Elution profiles of products of digestion of proteoglycan by human cathepsin B1 after overnight incubation under the conditions described for Fig. 1 (———), and after a further overnight incubation in the presence of 1.1 nmole (— · —) and yet another with 3.3 nmoles (— — —) of fresh cathepsin B1 per ml. The digestion with papain (3.75 nmoles/ml) was in the same buffer, but at 65 °C overnight (· · · · · ·).

Fig. 4. Rates of release of chondroitin sulphate from human embryonic cartilage in the presence of cathepsins B1 and D. Tissue (50 mg) depleted of lysosomal enzymes (see Methods) was incubated in 0.5 ml 0.1 M sodium acetate buffer, for 0–5 h at 37 °C with cathepsin B1 (1.4 nmole/ml) at pH 5.0 (■) or 6.0 (●). Samples were also incubated with 0.16 nmole cathepsin D/ml at pH 5.0 (□), or 8.0 nmoles cathepsin D/ml at pH 6.0 (○). Control samples were incubated without added enzyme (▲). The release of polysaccharide was measured turbidimetrically with chondroitin sulphate as standard[2] and expressed as a percentage of the total polysaccharide present in the tissue (measured turbidimetrically after digestion with papain). The values given are means of duplicate samples of each time.

than that resulting from prolonged treatment with cathepsin D; there remained only small amounts of material that were excluded by the gel (Fig. 3). The material from the viscometer was twice reincubated with fresh cathepsin B1 overnight, so that breakdown went to completion; the material now appeared as a single peak highly retarded on the gel, in the same position as material resulting from the action of papain on human proteoglycan. Again, there remained no material that was excluded from the gel.

Release of proteoglycan from cartilage by purified or endogenous enzymes

The rate of release of proteoglycan from foetal cartilage that had been washed free of proteolytic activity, during treatment with either cathepsin D or cathepsin B1 is shown in Fig. 4. At pH 5.0, cathepsin D was seen to be approximately 8 times as effective as cathepsin B1 on a molar basis; it was 5–10 times less effective at pH 6.0 (Fig. 5). Cathepsin B1 was approximately 3 times as active as cathepsin D on human cartilage under these conditions. However, during 100 h incubation at pH 5.0 cathepsin D (3.8 nmoles/ml) caused the release of 60% of the precipitable uronic acid in adult human cartilage (Dingle, J. T., unpublished results). The different pH dependences of the degradative activity of the two enzymes acting on ^{35}S-labelled cartilage are seen clearly in Fig. 6, where the effect of pH on the autolytic release of material from cartilage that had not been washed free of endogenous lysosomal proteases is

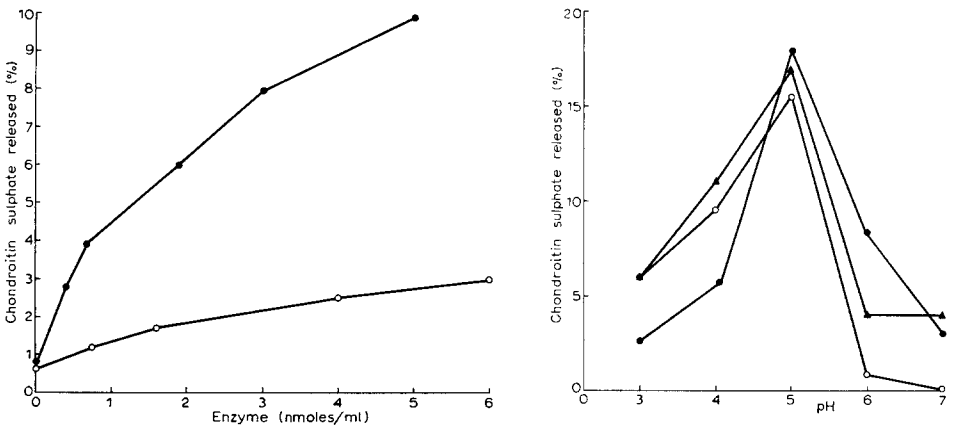


Fig. 5. Effect of concentration of cathepsins B1 and D on the release of polysaccharide from human foetal cartilage. Experiments were made at pH 6.0 for 2 h, as described in Fig. 4, with varying concentrations of cathepsin B1 (●) and cathepsin D (○).

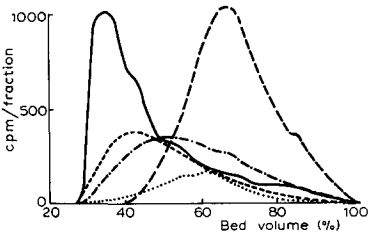
Fig. 6. Effect of pH on autolysis of foetal human articular cartilage, and on the action of cathepsins B1 and D on washed tissue. Duplicate 50 mg samples of ^{35}S-labelled cartilage (containing approx. 10^6 c.p.m.) were allowed to autolyse in 0.5 ml 0.2 M sodium acetate or phosphate buffers over the pH range 3–7 for 2 h at 37 °C in the presence of 0.1% Triton X-100, 1 mM dithiothreitol and 1 mM EDTA (▲). Other samples were washed to remove endogenous lysosomal enzymes before being incubated in the same manner, but in the presence of purified cathepsin B1 (2.8 nmoles/ml) (●), or cathepsin D (0.16 nmole/ml) (○). The release of proteoglycan fragments was measured as the appearance of ethanol-precipitable radioactivity in the buffer, and expressed as a percentage of the total precipitable activity initially present in the tissue.

also shown; the greatest release of labelled material occurred at pH 5.0, but a certain amount of activity remained towards neutral pH.

Effect of cathepsin D on molecular size of proteoglycan in cartilage

Foetal human articular cartilage was labelled with [^{35}S]sulphate *in vitro* and washed free of lysosomal enzymes (see Methods). Proteoglycan (as light protein-polysaccharide[18]) was prepared from the tissue without further treatment, and also from samples that had been incubated under control conditions and samples that had been incubated with cathepsin D. The preparations were run on Sepharose 2B, as was the proteoglycan released into the incubation media.

Proteoglycan prepared from washed cartilage before treatment with cathepsin D was largely excluded from the gel (Fig. 7). Material released into the medium from the cartilage during treatment with the enzyme, however, was well retarded and contained no excluded material. Material released during a control incubation without exogenous enzyme was slightly retarded, probably because of the action of residual traces of lysosomal enzymes in the tissue and the selective diffusion of low molecular weight material from the cartilage. Light proteinpolysaccharide remaining in cathepsin D-treated cartilage was well retarded on the gel, although it contained material of a rather higher molecular weight than that which was released into the medium. Proteoglycan remaining in cartilage incubated without cathepsin D was slightly retarded on the gel, probably because of the action of residual traces of lysosomal enzymes.



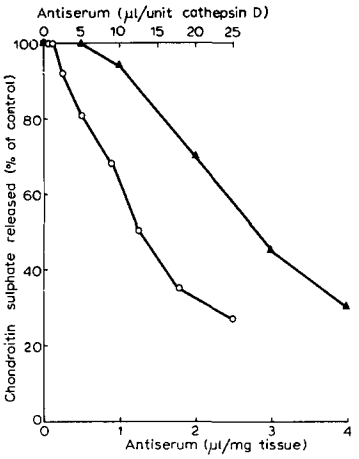


Fig. 7. Sepharose 2B gel chromatography of products of digestion of cartilage with cathepsin D. Gel chromatographic profiles of ^{35}S-labelled material released from human cartilage during treatment with cathepsin D (– – –), during the control incubation in buffer alone (— · —) and of a proteoglycan preparation from untreated cartilage (———). Also shown are the profiles of material remaining in the tissues after incubation with cathepsin D (· · · · · ·) and after incubation of the cartilage in the absence of added enzyme (· · ·). Tissue (150 mg) was incubated for 8 h in 2.0 ml of 0.2 M sodium acetate buffer, pH 5.0, with or without 0.16 nmole cathepsin D/ml.

Fig. 8. Immunoinhibition of breakdown of cartilage during autolysis and during treatment with cathepsin D. Foetal cartilage (100 mg) was allowed to autolyse, or to be degraded by exogenous cathepsin D, at pH 5.0 and 37 °C, in the presence of varying amounts of sheep anti-(human cathepsin D) serum. The release of chondroitin sulphate, measured as precipitable uronic acid[26], is expressed as a percentage of that in the presence of buffer and normal serum only. Autolysis (▲) was in 0.2 M sodium acetate buffer containing 0.1% Triton X-100, for 4 h. The incubation mixtures (1.0 ml) contained 0.40 ml of sheep serum containing varying amounts of antiserum. Treatment with cathepsin D (○) (4.2 units, or 0.16 nmole/ml) was as described in Fig. 4, but in the presence of 25 μl of serum containing varying amounts of antiserum, in a total volume of 1 ml. The release (○) is expressed as a percentage of that in the buffer containing only normal sheep serum, and values are means of triplicate estimations of the cetylpyridinium chloride-precipitable uronic acid[25].

Immunoinhibition of cartilage breakdown

When autolysis of foetal cartilage (100 mg) was carried out in the presence of sheep anti-(human cathepsin D) serum (4 μl/mg tissue), 70% inhibition of release of uronic acid-containing material was observed (Fig. 8). The antiserum also inhibited the action of the purified enzyme on washed cartilage, 70% inhibition occuring with 25 μl antiserum per unit of cathepsin D.

Unfortunately, comparable experiments could not be made with cathepsin B1, as the specific antisera raised by injection of this protein do not react with the active enzyme.

DISCUSSION

The present experiments demonstrate that both cathepsin D and cathepsin B1 are capable of degrading purified human proteoglycans, though to different degrees. The products resulting from the action of cathepsin B1 were shown by gel chromatography to be similar in size to those resulting from the action of papain, and would

therefore contain a single polysaccharide chain per molecule[27]. This is not altogether surprising, since cathepsin B1 and papain have similar specificities towards the peptide bonds of the oxidised B chain of insulin[28]. Cathepsin D, on the other hand, produced fragments which were similar in size to those resulting from the action of trypsin, which is thought to reduce proteoglycans to products containing two polysaccharide chains[27,29]. The specificity of cathepsin D is not as wide as that of papain or cathepsin B1 [28]. Human proteoglycans have been shown to differ in their susceptibility to attack by cathepsin D from proteoglycans prepared from rabbit ear or embryonic chick limb bone cartilage. Products resulting from the action of cathepsin D on these latter proteoglycans were larger than those produced by trypsin and were estimated to contain an average of three polysaccharide chains[4]. On the other hand, it was found that the incubations required to reduce human cartilage to its final products were several times longer than those for the chicken and rabbit tissues[4].

Examination by gel chromatography of the material liberated during the treatment of washed human cartilage with purified cathepsin D showed that the proteoglycan had been considerably degraded as compared to that which could be extracted from the cartilage by high speed homogenisation before treatment. Proteinpolysaccharide from the cathepsin D-treated cartilage was found to be degraded, as compared with that of untreated tissue. The products were, however, still of higher molecular weight than those released into the incubation medium during treatment with the enzyme. Thus, cathepsin D appears capable of diffusing throughout cartilage matrix and causing breakdown of proteoglycans under suitable conditions of pH.

Cathepsins D and B1 were effective in causing the release of proteoglycans from human cartilage that had been washed free of endogenous lysosomal proteolytic enzymes. Cathepsin D showed a pH optimum for degradative activity at pH 5.0, with practically no activity remaining at neutral pH. The activity of cathepsin B1 was also greatest at pH 5.0, but extended into the neutral range. The pH optimum of the autolysis of cartilage was consistent with a major role of both cathepsins B1 and D in this process. Furthermore, the presence of a sheep anti-human cathepsin D antiserum during autolysis at pH 5.0 resulted in a substantial (70%) inhibition of release of polysaccharide from the cartilage. When autolysis was carried out in the presence of the reducing and chelating agents required for the activation of thiol proteinases, increased release was observed in the pH range 5–7. Dingle *et al.*[10] showed by use of pepstatin that adult human cartilage contains an enzyme or enzymes active in autolysis at pH 5–7 only in the presence of EDTA and cysteine. It was suggested that a thiol proteinase such as cathepsin B1 might be involved, and this possibility is now strengthened by the demonstration that pure cathepsin B1 has the capacity to degrade cartilage proteoglycan. Although cathepsin D is the more important enzyme in the autolytic degradation of human cartilage at pH 5, the relative importance of the enzymes in living tissue remains to be determined. In particular, the capacity of cathepsin B1 to reduce the proteoglycan to small fragments, and its action on collagen[31] suggest that it may have an important role in the final intracellular stage of the catabolism of cartilage macromolecules.

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