A method was developed for generating soluble, active "aggrecanase" in conditioned media from interleukin-1-stimulated bovine nasal cartilage cultures. Using bovine nasal cartilage conditioned media as a source of the aggrecanase enzyme, an enzymatic assay was established employing purified aggrecan monomers as a substrate and monitoring specific aggrecanase-mediated cleavage products by Western analysis using the monoclonal antibody, BC-3 (which recognizes the new N terminal, ARGS, on fragments produced by cleavage between amino acid residues Glu372 and Ala374). Using this assay we have characterized cartilage aggrecanase with respect to assay kinetics, pH and salt optima, heat sensitivity, and stability upon storage. Aggrecanase activity was inhibited by the metalloprotease inhibitor, EDTA, while a panel of inhibitors of serine, cysteine, and asparagine proteinases had no effect, suggesting that aggrecanase is a metalloproteinase. Sensitivity to known matrix metalloproteinases had no effect, indicating that aggrecanase plays an important role in human aggrecan catabolism.

The aggregating cartilage proteoglycan, aggrecan, along with type II collagen, is responsible for the mechanical properties of articular cartilage. Aggrecan molecules are composed of two N-terminal globular domains, G1 and G2, which are separated by an interglobular domain (IGD), followed by a long central glycosaminoglycan (GAG) attachment region and a C-terminal globular domain, G3 (1–2). These aggrecan monomers interact through the G3 domain with hyaluronic acid and link protein to form large molecular weight aggregates which are trapped within the cartilage matrix (3–5). Aggrecan provides normal cartilage with its properties of compressibility and resilience, and is one of the first matrix components to undergo measurable loss in arthritis. This loss appears to be due to an increased rate of aggrecan degradation that can be attributed to proteolytic cleavage within the IGD of the core protein.

Cleavage within this region generates large C-terminal, GAG-containing aggrecan fragments lacking the G1 domain which are unable to bind to hyaluronic acid and thus diffuse out of the cartilage matrix.

Two major sites of proteolytic cleavage have been identified within the IGD: one between amino acid residues Asn371 and Phe342 and the other between amino acid residues Glu373 and Ala374. Matrix metalloproteinases (MMP-1, -2, -3, -7, -8, -9, and -13) have been shown in vivo to cleave within the IGD predominately at the Asn371-Phe342 site (6–10). Identification of G1 fragments formed by cleavage at the Asn371-Phe342 site within human articular cartilage (7, 11) and in synovial fluids (12) suggest a role for MMPs in proteoglycan degradation in vivo.

The second cleavage site was first described a number of years ago based upon identification of aggrecan fragments with an ARGS N terminus (13–15), however, the enzyme responsible for cleavage at the Glu373-Ala374 bond has not yet been identified. This uncharacterized activity has been given the name "aggrecanase" based on its ability to cleave the aggrecan core protein. Four other potential aggrecanase sites have been identified within the C-terminal region of aggrecan between G3 and G4 (14, 16), although the Glu373-Ala374 cleavage site within the IGD has been most widely studied. C-terminal fragments with the N terminus, ARGSV . . . , formed by cleavage between amino acid residues Glu372 and Ala374 have been identified in media from chondrocyte monolayer and cartilage explant cultures undergoing matrix degradation (13–19). This sequence was found to be present on a number of different size fragments, indicating that aggrecanase was cleaving at the Glu373-Ala374 bond within the IGD to generate products with a single N terminus which possessed variable C termini. N-terminal sequence analyses have identified aggrecan fragments in synovial fluids of patients with osteoarthritis, inflammatory joint disease, and joint injury (20, 21) which have the ARGSV N terminus, and G1 fragments have also been identified within the cartilage matrix with the NITEGE C terminus (11) indicating that aggrecanase plays an important role in human aggrecan catabolism.

Although evidence for induction of cleavage at the aggrecanase site within the IGD has been demonstrated in a number of in vivo tissue and cell culture studies (13–19), attempts to identify aggrecanase proteolytic activity in culture media or cell/tissue extracts from these models have been largely unsuccessful. Recent work using an artificial recombinant protein comprising the IGD sequence of aggrecan, rAgg1, as a substrate, has identified activity in media from retinoic acid-stimulated rat chondrosarcoma cell cultures which cleaves at the Glu373-Ala374 bond (22). However, the protease responsible for this cleavage remains unidentified and uncharacterized.

In this paper we describe the generation of soluble, active aggrecanase activity in conditioned media from interleukin-1
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(IL-1)-stimulated bovine nasal cartilage. Using this source of aggrecanase, we have developed an enzymatic assay employing purified native aggrecan monomers as the substrate and detection of specific products of aggrecanase-mediated cleavage by Western analysis with the monoclonal antibody, BC-3 (23), which recognizes the new N terminus ARGSV ... fragment producing by cleavage at the aggrecanase site. We have performed initial enzymatic characterization of the soluble cartilage aggrecanase activity generated in response to IL-1. The work described herein provides a method for generating soluble aggrecanase activity and an assay for monitoring this activity which should serve as important tools to enable the isolation, purification, and molecular characterization of the enzyme.

EXPERIMENTAL PROCEDURES

Materials— Dulbecco’s modified Eagle’s medium and fetal bovine serum were from Life Technologies, Inc. (Grand Island, NY). The IL-1 used was a soluble, fully active recombinant human IL-1β produced as described previously (24). The specific activity was 1 × 10^7 units/mg of protein, with 1 unit being defined as the amount of IL-1 that generated half-maximal activity in the thymocyte proliferation assay. Antibody ammonium were from Boehringer-Mannheim (Indianapolis, IN). The allopurinol (TIMP-1) was from Calbiochem (Cambridge, MA). The Seikuguku (Kogyo, Japan). The full-length bovine tissue inhibitor of metalloproteinases-1 (TIMP-1) was from Calbiochem (Cambridge, MA). The protease inhibitors, antipain dihydrochloride, aprotinin, bestatin, chymotrypsin, E-64, EDTA, leupeptin, Pefabloc, pepstatin, and phosphoramidon were from Boehringer-Mannheim (Indianapolis, IN). The hydroxycarbonyl acid MMP inhibitors, BB-16 ((2S,3R)-2-methyl-3-(2-methylpropyl)-1-((N-hydroxy-4)-d-oxy-1-tirolese-N-methyl amide), XS309 (3S-[3R,2R,2’S]-hexahydro-2-[2-[2-(hydroxymino-1-methyl-2-oxoethyl]-4-methyl-1-oxopentyl]-N-methyl-3-pyridazincarboxamide), and SA751 (N-[1(R)-Carboxymethyl]-a-S)-4-(phenyl-3-butylnlyglycyl-L-phenylalanine, N-methylamide) were synthesized at DuPont Pharmaceuticals as described previously (25, 26). BB-16 and XS309 are potent nanomolar inhibitors of a number of MMPs, including MMP-1, -2, -3, -8, and -9, while SA751 is a selective MMP-8 inhibitor which should serve as important tools to enable the isolation, purification, and molecular characterization of the enzyme.

Aggrecanase Enzymatic Assay—Casein labeled with resorufin (43.5 μM) was incubated with protease-containing media in a final volume of 200 μl of 50 mM Tris-HCl, 5 mM CaCl₂, pH 7.8 at 37 °C as described previously (29). The reactions were stopped and unclipped substrate was added to achieve trichloroacetic acid to a final concentration of 5%. Samples were filtered through a 96-well filtration plate (Millipore Corp. Bedford, MA) and filters collected and neutralized by addition of 2 M Tris to each well. The absorbance was then read at 575 nm. The concentration of the resorufin-labeled peptide in the filtrate, as determined from a standard curve, was used as a measure of proteolytic activity. Some conditioned medium samples were assayed in the presence of 2 μM 4-aminohippuric acid (APMA) to activate latent matrix metalloproteinases. With some samples EDTA was included to inhibit metalloproteinase activity or XS309 was included to inhibit matrix metalloproteinase activity.

Analysis of Aggrecan Catabolites—For analysis of aggrecan fragments generated by specific cleavage at the Glu773-Ala775 site, proteoglycans and proteoglycan metabolites were enzymatically deglycosylated with chondroitinase ABC (0.1 units/μg of GAG) for 2 h at 37 °C in the presence of 2 mM 4-aminophenylmercuric acetate (APMA) to activate latent matrix metalloproteinases. With some samples EDTA was included to inhibit metalloproteinase activity or XS309 was included to inhibit matrix metalloproteinase activity.

Gelatin Zymography—Culture media were diluted 1:20 with water and 15 μl mixed with an equal volume of sample buffer (0.5 mM Tris-HCl, pH 6.8, 4% SDS, 0.005% bromophenol blue, 20% glycerol) (Novex, San Diego, CA), incubated at room temperature for 10 min, and run on a 10% SDS-PAGE gel containing 0.1% gelatin (0.1 mg/ml Tris, 2 μg gelatin, 35 mM SDS, pH 8.3) for 90 min at 125 volts. The gels were renatured in 2.5% Triton X-100 in water for 30 min at room temperature and then incubated at 37 °C overnight in developing buffer (50 mM Tris, 0.2 mM NaCl, 5 mM CaCl₂, 0.02% Brij 35, pH 7.6). After incubation, gels were stained in 0.25% Coomassie Brilliant Blue R-250 for 4 h at room temperature and destained in distilled water containing 30% methanol and 10% glacial acetic acid to reveal zones of lysis within the gelatin matrix. EDTA (5 mM), E64 (10 μg/ml), pepstatin (1 μg/ml), or benzamidine HCl (10 mM) or XS309 (1 μM) were added to the developing buffer to identify which classes of proteinases were responsible for lysis of the gelatin.

Aggrecanase Enzymatic Assay—Casein labeled with resorufin (43.5 μM) was incubated with protease-containing media in a final volume of 200 μl of 50 mM Tris-HCl, 5 mM CaCl₂, pH 7.8 at 37 °C as described previously (28). The reactions were stopped and unclipped substrate was added to achieve trichloroacetic acid to a final concentration of 5%. Samples were filtered through a 96-well filtration plate (Millipore Corp. Bedford, MA) and filters collected and neutralized by addition of 2 M Tris to each well. The absorbance was then read at 575 nm. The concentration of the resorufin-labeled peptide in the filtrate, as determined from a standard curve, was used as a measure of proteolytic activity. Some conditioned medium samples were assayed in the presence of 2 μM 4-aminohippuric acid (APMA) to activate latent matrix metalloproteinases. With some samples EDTA was included to inhibit metalloproteinase activity or XS309 was included to inhibit matrix metalloproteinase activity.
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RESULTS

Aggrecanase Generation in Bovine Nasal Cartilage Culture—Incubation of bovine nasal cartilage with 500 ng/ml IL-1 for 6 days, with media replaced every 2 days, induced the degradative cleavage and release of aggrecan from the cartilage (Fig. 1). By monitoring the amount of GAG remaining in the cartilage at the end of the incubation, percent release of GAG from the tissue was determined at each time period; greater than 95% release of GAG was achieved by day 6. Cartilage was then incubated with IL-1 for an additional 10–12 days without media changes to allow aggrecanase to accumulate in the media. To evaluate the time course of aggrecanase activity generation, samples of conditioned media were taken from cultures at various times during incubation with IL-1 and evaluated for aggrecanase activity. Incubation of this conditioned media with aggrecan substrate for 4 h resulted in the generation of fragments cleaved at the Glu373-Ala374 site as detected by BC-3 Western blotting. After an additional 10–12 days of incubation with IL-1, incubation of day 6 media with 500 nM aggrecan substrate for 4 h resulted in the formation of a BC-3-reactive, aggrecanase-generated product of ~250 kDa. Aggrecanase activity accumulated in the media as the enzyme activity was monitored from days 6 to 16. In media from cultures stimulated for 10 days or greater, additional minor BC-3-reactive bands that migrated between 100 and 250 kDa were detected.

Aggrecanase Enzymatic Assay Development—In order to establish appropriate assay conditions and evaluate aggrecanase kinetic properties, the effect of incubation time, enzyme concentration, and substrate concentration on product formation were evaluated. Linearity of product formation was observed up to 4 h (Fig. 3A), and over enzyme concentrations between 25 and 80 μl of conditioned media (Fig. 3B). Aggrecanase activity appeared to approach saturation with respect to aggrecan substrate at 500–1000 nM (Fig. 3C). However, increasing viscosity precluded use of higher substrate concentrations.

Stability—Aggrecanase activity was found to be stable during storage at −70, −20, or 4 °C in culture media; greater than 90% of the activity was recovered after 14 days or longer storage at −70, −20, and 4 °C. Activity was also stable to repeated freeze-thaw cycles of −70 to 4 °C. Heating at 42 °C for 15 min did not affect activity, but activity was completely lost following 15 min heating at 56 °C or above.

Characterization of Aggrecanase Activity—Using the assay conditions defined above, we studied the salt and pH optimum of crude aggrecanase present in conditioned medium. Optimal activity was achieved with 100 mM NaCl. At 250 mM NaCl, activity was decreased by ~50% and was completely lost at 500 mM NaCl or higher (Fig. 4). The pH optimum for aggrecanase was found to be at 7.5 (Fig. 5). However, a rather broad pH range between 6.5 and 9.5 supported greater than 75% of the activity seen at the pH optimum.

Effect of Protease Inhibitors—Aggrecanase activity was inhibited by the metalloprotease inhibitor, EDTA, while a panel of inhibitors of serine, cysteine, and aspartic proteinases had no effect (Table I). The ability of the endogenous inhibitor of matrix metalloproteinase, TIMP-1, to block aggrecanase activity was investigated using full-length recombinant bovine TIMP-1. TIMP-1 caused a concentration-dependent inhibition of aggrecanase activity (Fig. 6). The IC50 was estimated from the concentration-response curve to be 210 nM. However, TIMP-2 at concentrations up to 10 μM was inactive against aggrecanase (data not shown).

Effect of Peptidase Inhibitors—Aggrecanase activity was inhibited by the metalloprotease inhibitor, EDTA, while a panel of inhibitors of serine, cysteine, and aspartic proteinases had no effect (Table I). The ability of the endogenous inhibitor of matrix metalloproteinase, TIMP-1, to block aggrecanase activity was investigated using full-length recombinant bovine TIMP-1. TIMP-1 caused a concentration-dependent inhibition of aggrecanase activity (Fig. 6). The IC50 was estimated from the concentration-response curve to be 210 nM. However, TIMP-2 at concentrations up to 1 μM was inactive against aggrecanase (data not shown).

The peptidic hydroxamates, BB-16 (25) and XS309 (26), which are potent, synthetic inhibitors with broad specificity against MMPs were tested for their ability to block aggrecanase activity (Table II). BB-16 resulted in a concentration-dependent inhibition of aggrecanase activity, while XS309 was inactive at concentrations up to 10,000 nM. Previous studies from our laboratory suggested that although MMP-8 (neutrophil collagenase) has the ability to cleave native aggrecan at the Glu373-Ala374 bond, it does not represent the cartilage aggrecanase (31). To confirm this hypothesis we also evaluated

* M. D. Tortorella, M. A. Pratta, and E. C. Arner, unpublished data.
SA751 (26), a potent, selective synthetic MMP-8 inhibitor, for its ability to inhibit aggrecanase activity by incubating with aggrecan substrate (500 nM) for 4 h at 37 °C and evaluating products by BC-3 Western blot analysis. Media quenched with EDTA prior to incubation with substrate served as assay blanks (lanes labeled B at each time point).

To ascertain whether aggrecanase activity was associated with known metalloproteinase activities, gelatinolytic and caseinolytic activities were assayed in medium from IL-1-stimulated cartilage. Multiple bands of gelatinolytic activity were observed in conditioned medium by gelatin zymography. All of these gelatinases were shown to be metalloproteases by the complete loss of gelatinolytic activity when the zymogram was incubated with EDTA (Fig. 7), whereas E-64, pepstatin, or benzamidine hydrochloride (which inhibit the cysteine, aspartate, and serine protease classes, respectively) had no effect on gelatinase activity under similar conditions (data not shown).

In contrast to its lack of effect on aggrecanase activity, XS309 resulted in a complete inhibition of the gelatinolytic activity (Fig. 7). Similarly, caseinolytic activity was also detected in the conditioned media. Both active and APMA-activated caseino-
lytic activity were blocked by EDTA (Table III), indicating that this activity was due to a metalloproteinase(s). Furthermore, this caseinolytic activity was completely blocked by XS309, the MMP inhibitor that was ineffective against aggrecanase.

**Discussion**

Although several studies suggest that cleavage of the aggrecan core protein at the Glu373-Ala374 bond plays a key role in cartilage matrix degradation, the protease responsible for this cleavage remains unidentified. Therefore, our current study had three main goals: 1) to establish a method for generating active aggrecanase; 2) to develop an assay to assess and follow this activity; and 3) to characterize aggrecanase enzymatic activity.

Stimulation of chondrocyte cultures with retinoic acid or bovine nasal cartilage with IL-1 for short time periods has been shown to result in the production of aggrecan fragments formed...
by cleavage at the Glu$^{372}$-Ala$^{374}$ bond (18, 32, 33). However, aggrecanase activity as measured by cleavage of exogenous aggrecan was not detected in media from these cultures (34). We had previously found that induction of MMP-3 in cartilage cultures by IL-1 resulted in the elution of only the inactive zymogen into the culture media at early time points, although both the zymogen and active forms of the enzyme were present within the matrix (35). Only after depletion of aggrecan from the matrix or in the presence of an active-site inhibitor was the active form of MMP-3 protein detected in the culture media, suggesting that the active enzyme was bound to a matrix component, possibly aggrecan. We reasoned that this might also be the case for aggrecanase. Therefore, in an attempt to generate and release active aggrecanase from bovine nasal cartilage, we first stimulated the cartilage to deplete the aggrecan from the matrix and then restimulated and monitored the media for the presence of aggrecanase activity. This procedure resulted in the generation of readily detectable aggrecanase activity in conditioned media. In contrast to MMP-3, aggrecanase was present in the conditioned media as the active form at days 2 and 4 of incubation with IL-1. However, products generated during the enzymatic assay were much easier to detect following depletion of the matrix when background BC-3-reactive fragments were no longer present in the conditioned media.

The conditioned media exhibited aggrecanase activity (i.e., cleavage of aggrecan at the Glu-Ala bond) without activation. In fact, incubation with APMA or chymotrypsin, which are frequently used to activate pro-MMPs, did not increase this activity. In addition, XGO76, an inhibitor of pro-MMP activation, which is effective in blocking aggrecanase-mediated cleavage in IL-1-stimulated bovine explant cultures (33), did not inhibit aggrecanase in the enzymatic assay, indicating that aggrecanase is not being activated in some manner during the assay. Although we cannot rule out that the active aggrecanase could be bound to matrix molecules, specific binding and orientation within the matrix is not required, since we observe activity using isolated aggrecan substrate in solution. Thus, aggrecanase generated in conditioned media from IL-1-stimulated bovine nasal cartilage is a soluble, activated enzyme, free of association with the chondrocyte cell surface. These characteristics are consistent with those reported in rat chondrosarcoma cell cultures stimulated with retinoic acid, where activity was detected in media using an artificial recombinant aggrecan interglobular domain protein substrate (22). In contrast to earlier studies (34), a recent publication demonstrated the ability of membranes isolated from stimulated chondrocytes to generate aggrecan fragments formed by cleavage at the Glu$^{372}$-Ala$^{374}$ bond (36). This recent identification of aggrecanase activity associated with chondrocyte membranes, in addition to our demonstration of soluble activity in culture media, open the possibility that aggrecanase may be an integral membrane protein that is subsequently cleaved to a soluble form or that it may be a soluble enzyme that is associated with a cell surface-binding protein on the chondrocyte membrane.

Although other matrix metalloproteases are induced in the aggrecanase-containing conditioned media from IL-1-stimulated cartilage, none of these appear to be responsible for the BC-3-reactive fragments produced by cleavage at the Glu$^{372}$-Ala$^{374}$ bond. Studies using purified enzymes have shown that MMP-1, -2, -3, -7, -8, and -9 cleave at the Asn$^{341}$-Phe$^{342}$ bond and do not readily cleave at the Glu$^{372}$. Ala$^{374}$ site (6–10). Furthermore, we have shown that 1 μM XS309, a potent MMP inhibitor with Ki values in the nanomolar range against MMP-1, -2, -3, -8, and -9 that was inactive at concentrations up to 10 μM in inhibiting aggrecanase, completely blocked all gelatinolytic activities in aggrecanase-containing media. These data indicate that aggrecanase does not cleave gelatin and that aggrecanase activity is not represented by these gelatinolytic MMPs induced in response to IL-1 stimulation. Caseinolytic activity, detected in the conditioned media, was inhibited almost completely by 1 μM XS309. Since at 1 μM XS309 does not inhibit aggrecanase, these data suggest that aggrecanase does not cleave casein and that the caseinolytic activity generated in response to IL-1 does not represent aggrecanase. Interestingly, XS309, which completely blocked both the gelatinolytic and caseinolytic activity generated in response to IL-1, was found to be inactive in blocking IL-1-induced aggrecan degradation in cartilage explant cultures, while BB-16, which is active against aggrecanase enzymatic activity, was effective (33).

The substrate in the enzymatic assays used to determine Ki values of the synthetic MMP inhibitors against the various MMPs was a fluorogenic peptide substrate. We have not determined the Ki values for these compounds using the natural substrates for the different MMPs or using aggrecan, to compare with the Ki values determined using the peptide substrate. Therefore, the possibility that this difference in substrate may confound the comparison of the potency of these compounds against MMPs with their potency in the aggrecanase assay, where native aggrecan is used as the substrate, must be considered. However, when XS309 and BB-16 were evaluated for inhibition of MMP-3 using the native aggrecan substrate, these compounds exhibited potency in the nanomolar range as was the case using the peptide substrate.

Using BC-3 Western blot analysis to monitor aggrecan fragments produced by cleavage at the Glu$^{372}$-Ala$^{374}$ bond, we have developed an enzymatic assay specific for aggrecanase-generated products. Although Western blot analysis does not provide a particularly rapid means of product evaluation, quantitation is possible, thus enabling characterization of aggrecanase activity. This assay was used to assess product formation with respect to incubation time and enzyme and substrate concentration dependence. Aggrecanase displayed a sensitivity to salt, with activity being lost at NaCl concentrations above 500 mM. A broad pH optimum extending into the alkaline range was observed, although maximum activity was seen at pH 7.5. Aggrecanase was also sensitive to elevated temperature with the activity being lost after treatment at 56 °C.

Inhibitor studies indicate that aggrecanase is a metalloendopeptidase as it is inhibited by the metalloprotease inhibitor EDTA, but not by inhibitors of the cysteine, serine, or aspartyl protease classes. Inhibition by TIMP-1 opens the possibility that this protease may be a member of the matrix metalloproteinase family of enzymes. However, the IC50 for inhibition of aggrecanase by TIMP-1 is more than 100-fold higher than that reported for MMPs (37, 38). In addition, we were not able to obtain inhibition with TIMP-2 at concentrations up to 1 μM. During the time this manuscript was in review, similar results were reported for inhibition of aggrecanase activity from chondrocytes using a recombinant aggregan interglobular domain substrate (39). Aggrecanase could represent a member of the MMP family that differs significantly from other MMPs in its interaction with TIMP-1 or which may lack the C-terminal region that has been shown to be important in binding to TIMP for some MMPs (40). Alternatively, aggrecanase may be a member of a closely related non-MMP class of metalloproteases.

Although the MMP inhibitor, BB-16, is capable of inhibiting

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3 E. C. Arner and M. D. Tortorella, unpublished data.

4 E. C. Arner and M. A. Pratta, unpublished data.
the proteolytic activity of aggrecanase, not all MMP inhibitors are effective in blocking this activity. In addition, some hydroxamates have been shown to inhibit other metalloproteases, such as TNF converting enzyme (41, 42), which is a member of the family of mammalian adamalysins or ADAMs (a disintegrin and metalloproteinase domain) proteins (43). Our laboratory has recently demonstrated that atrolysin C, a snake venom metalloproteinase with homology to the ADAM proteins is capable of cleaving aggrecan at the Glu373-Ala374 bond and at site(s) consistent with these fragments having undergone cleavage both through day 6 and 4. Lark, M. W., Gordy, J. T., Weidner, J. R., Ayala, J., Kimura, J. H., Williams, J. A. (1995) J. Biol. Chem. 270, 169–181.

Isolation and purification of the aggrecanase enzyme requires a tissue source of the protease and an enzymatic assay to follow activity during purification. In the studies reported herein, we have developed a method for generating soluble aggrecanase activity from bovine nasal cartilage, performed initial characterization of this protease, and developed an assay for monitoring this activity which should lead to the isolation of the aggrecanase enzyme.

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