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Identification of Aggrecanase Activity in Medium of Cartilage Culture

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Erosion of cartilage is a major feature of joint diseases, *i.e.*, osteoarthritis and rheumatoid arthritis, which leads with time to a loss of joint function. Proteolytic cleavage of the aggrecan core protein is a key event in the progress of these joint diseases. Aggrecan degradation has been believed to be mediated by a putative proteinase, aggrecanase. We identified aggrecanase activity in conditioned medium from explant culture of bovine nasal cartilage stimulated by retinoic acid. The activity was partially purified more than 10,000-fold. The enzyme cleaves at the aggrecanase site (Glu^{373}-Ala^{374}) but not at the MMP site (Asn^{341}-Phe^{342}) in the interglobular domain of the aggrecan. It also cleaves at Glu^{1971}-Leu^{1972}, which is located in the gap region in the chondroitin sulfate attachment region prior to the aggrecanase site. The enzyme is a typical Ca^{2+}-dependent metalloproteinase with a unique salt-dependency and is inhibited by several hydroxamate-based inhibitors for matrix metalloproteinases. Heparin and chondroitin sulfate inhibited the enzyme in a dose-dependent manner, suggesting that the large carbohydorate in aggrecan is important for substrate recognition by aggrecanase.

Key words: aggrecan, aggrecanase, cartilage culture, joint disease, matrix metalloproteinase.

The ability of cartilage to deform reversibly after repetitive mechanical loading is due in large measure to the hyaluronan-proteoglycan complex trapped within the type II collagen network. Aggrecan, a large aggregating proteoglycan, is the most abundant proteoglycan in the soft tissues of the joint, which include the articular and meniscal cartilage and the supporting intraarticular ligaments (*1, 2*). Aggrecan provides the compressive and tensile strength that is characteristic of cartilage. It consists of an extended protein core, which can be subdivided into several structural regions: the N-terminal globular G1 and G2 domains, the keratan sulfate (KS) and chondroitin sulfate (CS) attachment region, and the C-terminal globular G3 domain (*3*). The KS and CS chains of aggrecan effectively concentrate the negative charges that maintain the expanded tissue-volume characteristics of cartilage. The G1 domain of aggrecan interacts with hyaluronan and link protein to form large aggregates that are immobilized in the tissues with type II collagen fibrils.

The destruction of joint cartilage is of central importance in human arthritic diseases, *i.e.*, osteoarthritis (OA) and rheumatoid arthritis (RA). Turnover of aggrecan is critically important to maintain extracellular matrix homeostasis in articular cartilage, because the loss of aggrecan from cartilage leads to a reduction in the hydration of the matrix and consequent reduction in compressive stiffness (*4*). Proteolytic cleavage of the aggrecan core protein within the interglobular domain (IGD) between the G1 and G2 domains is a key event in the progress of joint diseases. This cleavage results in the release of a large glycosaminoglycan (GAG)-containing aggrecan fragment which diffuses out of the cartilage matrix, while the G1 domain remains on hyaluronan in the matrix, causing irreversible denaturation of cartilage (*3*). The initial investigation of naturally occurring degradation showed that the IGD was cleaved predominantly at one site. The major cleavage site was identified as Glu^{373}-Ala^{374} based on the analyses of aggrecan fragments released from cartilage explant culture and primary cultured chondrocytes (*5-9*). The same cleavage site was also identified in aggrecan fragments in synovial fluids of patients with various joint diseases (*10-12*). These observations suggest that aggrecan breakdown is catalyzed *in vivo* by a glutamyl-endopeptidase. Since no known mammalian proteinase has specificity for cleavage at the position after glutamic acid, it is suggested that a novel proteinase, aggrecanase, is involved in the degradation of aggrecan in arthritis (*13*).

In this study, we identified and characterized aggrecanase activity in the conditioned medium of cartilage culture. The enzyme is a Ca^{2+}-dependent metalloproteinase, and is inhibited by GAGs and several hydroxamate-based inhibitors for matrix metalloproteinases (MMPs).

MATERIALS AND METHODS

Inhibitors—Actinonin, captopril, 1,10-phenanthroline

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Abbreviations: ADAM, a disintegrin and metalloproteinase; CS, chondroitin sulfate; GAG, glycosaminoglycan; IGD, interglobular domain; IL-1, interleukin-1; KS, keratan sulfate; MMP, matrix metalloproteinase; OA, osteoarthritis; RA, rheumatoid arthritis; SVMP, snake venom metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

and thiorphan were from Sigma (St. Louis, MO, USA). All hydroxamate-based metalloproteinase inhibitors, AG3340 (*14*), BB94 (*14, 15*), and CT1746 (*16*), were synthesized at the Chemical Research Laboratories, Sankyo. Recombinant human tissue inhibitor of metalloproteinase-1 (TIMP-1) and TIMP-2 were from Fuji Chemical Industries (Takaoka).

Preparation of Aggrecan—Aggrecan was extracted from bovine nasal cartilage in 10 volumes (w/v) of 4 M guanidine hydrochloride, 50 mM sodium acetate, pH 6.0, 10 mM EDTA, 1 mM benzamidine hydrochloride, 1 mM phenylmethylsulfonyl fluoride, 0.1 M 6-amino-*n*-hexanoic acid, and 10 mM *N*-ethylmaleimide. Solubilized aggrecan was purified on a CsCl gradient centrifugation under both associative and dissociative conditions (*17*). Purified aggrecan was dialyzed against H_2O, lyophilized, and stored at 4°C until use.

Cartilage Explant Cultures—Bovine nasal cartilage was cultured as described previously with a slight modification (*7*). Cartilage was diced into small cubes and maintained as organ culture in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY) supplemented with 20 mM HEPES, pH 7.3, 100 unit/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, 1 μg/ml amphotericin B, insulin-transferrin-selenium (Sigma), and 0.03% (w/v) BSA. Cultures containing 5 g of cartilage in 30 ml of the medium were preincubated for 5 h, then 1 μM all-*trans* retinoic acid (Sigma) was added to the medium. After 24 h (day 1), gentamicin and amphotericin B were omitted, and the cultures were maintained with daily medium exchange. The conditioned media from day 3 to day 7 were pooled and stored at −40°C until use.

Partial Purification of Aggrecanase—All purification procedures were performed at 4°C. The pooled conditioned medium (1,200 ml) was thawed, filtered through 2-layers of gauze, then applied to a 200-ml Q-Sepharose FF (Amersham Pharmacia Biotech, Piscataway, NJ) column equilibrated with 20 mM Tris-HCl, pH 7.2, and 5 mM $CaCl_2$. Aggrecanase activity passed through the column. The active fractions (1,400 ml) were adjusted to 1 M NaCl and 0.5% (w/v) CHAPS, then applied to a 50-ml Zn-chelating Sepharose FF (Amersham Pharmacia Biotech) column equilibrated with buffer A (20 mM Tris-HCl, pH 7.2, and 0.5 M NaCl) containing 5 mM $CaCl_2$. After extensive washing with 50 ml of buffer A containing 0.5% CHAPS and 5 mM $CaCl_2$, 250 ml of buffer A containing 5 mM $CaCl_2$, and 50 ml of buffer A containing 35 mM imidazole and 1 mM $CaCl_2$, aggrecanase was eluted with buffer A containing 50 mM imidazole and 1 mM $CaCl_2$. The pooled active fractions (120 ml) were adjusted to 1.2 M $(NH_4)_2SO_4$ and applied to a 5-ml Ether Toyopearl 650S (TOSOH, Tokyo) column equilibrated with buffer B (20 mM Tris-HCl, pH 7.2, and 1 mM $CaCl_2$) containing 1.2 M $(NH_4)_2SO_4$. After washing the column with 15 ml of buffer B containing 1.3 M $(NH_4)_2SO_4$, aggrecanase activity was eluted with 50 ml of a linear gradient of 1.2–0.4 M $(NH_4)_2SO_4$ in buffer B. The active fractions from the column were dialyzed against buffer C (20 mM MES-NaOH, pH 6.5, 0.05% (w/v) Brij 35, 10 μM $ZnSO_4$, and 5 mM $CaCl_2$) containing 0.15 M NaCl, then applied to a Mono S HR 5/5 column (Amersham Pharmacia Biotech). Aggrecanase was eluted with 35 ml of a linear gradient of 0.2–0.7 M NaCl in buffer C at a flow rate of 1 ml/min using an FPLC system (Amersham Pharmacia Biotech).

Preparation of Neoepitope-Specific Antiserum—A neoepitope-specific antiserum, I19C, was prepared as described previously with a slight modification (*9*). Briefly, I19C was raised in rabbit against the amino acid sequence C-Nle-NITEGE coupled to keyhole limpet hemocyanin at the cysteine residue. This sequence represents the new C-terminal sequence of aggrecan catabolic products generated by the activity of aggrecanase (*6–12*). The specific antibodies were affinity purified by use of the immunizing peptide and used in subsequent immunoblotting experiments.

Aggrecanase Assay—The enzyme reaction was carried out in 50 μl of assay buffer (20 mM Tris-HCl, pH 7.2, 0.15 M NaCl, and 5 mM $CaCl_2$) containing 100 μg of aggrecan. After various periods of incubation with conditioned medium of cartilage culture or enzyme preparations at 37°C, the reaction was stopped by the addition of 2.5 μl of stop solution (0.72 M Tris-HCl, pH 8.0, 0.6 M sodium acetate, and 0.2 M EDTA). Samples were treated with 0.15 unit of chondroitinase ABC (Seikagaku Kogyo, Tokyo) and 0.1 unit of keratanase (Seikagaku Kogyo) at 37°C for 1 h (*5*). The deglycosylated samples were subjected to 2–15% gradient SDS-PAGE (Daiichi Kagaku, Tokyo) and stained with quick CBB staining solutions (Wako, Osaka). The gels were scanned by densitometer (GS-700, BIO-RAD, CA) to quantify the amounts of remaining intact aggrecan and to determine specific activities during purification of the enzyme. For immunological detection, gels were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore, MA), followed by Western analysis with I19C antiserum.

In some experiments, particle assay was used for quantitative measurements of the enzymatic activity as described previously (*18*) with a slight modification. In outline, polyacrylamide particles containing aggrecan were prepared (*18*), and the enzyme reactions were performed in 100 μl of 50 mM Tris-HCl, pH 8.0, 0.25 M NaCl, 5 mM $CaCl_2$, and 1.2 mg aggrecan particles at 37°C for 3 h. In determining the effect of TIMPs on the aggrecanase activity, the enzyme was preincubated with TIMPs at room temperature for 15 min. The reaction was stopped by adding EDTA to a final concentration of 10 mM. After brief centrifugation to remove the particles, 30 μl of the reaction supernatants containing GAGs released from the particles were added to 150 μl of 1,9-dimethylmethylene blue (Aldrich, Milwaukee, WI) dye solution. Absorbency at 525 nm was measured at 30 s after dye addition using whale chondroitin sulfate as a standard.

Identification of the Cleavage Sites in Aggrecan—Partially purified aggrecanase eluted from a Mono S column was incubated with 2 mg of aggrecan in 1 ml of assay buffer at 37°C for 16 h. After the reaction, samples were treated with 0.5 unit of chondroitinase and 0.2 unit of keratanase for 5 h. The deglycosylated samples were lyophilized and dissolved in 4 M guanidine hydrochloride and 20 mM sodium acetate, pH 6.0. Large fragments of aggrecan of over 200 kDa were separated from the smaller fragments by gel filtration using a Superose 6 HR 10/30 (Amersham Pharmacia Biotech) equilibrated with 4 M guanidine hydrochloride and 20 mM sodium acetate, pH 6.0. The fractions containing the large fragments were dialyzed against 20 mM sodium acetate, pH 6.0, transferred to PVDF membrane, then subjected to N-terminal sequencing. The

fractions containing smaller fragments were dialyzed against 20 mM sodium acetate, pH 6.0, lyophilized, and subjected to 7.5% SDS-PAGE. The fragments were transferred to PVDF membrane and stained with amide black. The regions containing the fragments were cut out and subjected to N-terminal sequencing. Amino acid sequence analysis was performed on a sequencer (PPSQ-10, Shimadzu) with on-line phenylthiohydantoin analysis.

RESULTS

Identification of Aggrecan-Degrading Activity in Cartilage Explant Culture—Following stimulation by retinoic acid, which is known to accelerate proteoglycan degradation in cultured cartilage, aggrecan-degrading activity in the conditioned medium was monitored at 24-h intervals. Purified bovine aggrecan was incubated with the conditioned medium in a buffer containing 2 mM Ca^{2+}, 2 mM Mg^{2+}, and 0.15 M NaCl to mimic physiological extracellular conditions, then the mixture was treated with chondroitinase and keratanase and analyzed by SDS-PAGE. Under these conditions, two cleavage products appeared from day 2 to day 7 after stimulation by retinoic acid, reaching maximum at day 4 and 5 (Fig. 1A). The same samples were also analyzed by Western blotting with I19C antiserum. An aggrecanase-derived fragment was generated in proportion to the induction of the degrading activity (Fig. 1C). No such activity was detected in the unstimulated culture medium (Fig. 1, B and D).

Partial Purification of Aggrecanase—The detected aggrecan-degrading activity was partially purified (Fig. 2). To facilitate the purification by minimizing the interaction of aggrecanase with aggrecan released into the conditioned medium, high concentrations of NaCl and CHAPS were added to the pooled fractions from the Q-Sepharose column. Then the fractions were loaded onto a Zn-chelating column, which retained the degrading activity but not the aggrecan. In all chromatographic steps, the degrading activities observed in Fig. 1 were always eluted in the same fractions. The activity was enriched over 10,000-fold from the conditioned medium after four-step purification using Q-Sepharose, Zn-chelating, Ether Toyopearl, and Mono S columns (Fig. 2A). SDS-PAGE analysis showed that the active fractions from the Mono S column mainly contained a 55-kDa protein (Fig. 2, B and C). Further analysis, however, revealed that the protein did not contribute to the aggrecanase activity (data not shown). The Mono S fraction, although not homogenous on SDS-PAGE, was used in the following experiments.

N-Terminal Amino Acid Sequencing of Aggrecan Core Fragments Generated by Aggrecanase—The aggrecan core fragments generated by the enzyme were analyzed by SDS-PAGE, and the N-terminal amino acid sequence of each fragment was determined as shown in Fig. 3. The enzyme mainly cleaved aggrecan at two sites, and resultant three fragments are illustrated in Fig. 3C. The enzyme cleaved at the aggrecanase site (Glu^{373}-Ala^{374}), generating fragments II (320 kDa) and IV (60 kDa). On the other hand, the enzyme did not cleave at the MMP site (Asn^{341}-Phe^{342}), which would generate the 55-kDa band (*9*). These results are consistent with the characteristics of aggrecanase, suggesting that the enzyme is distinct from the MMP family. The enzyme, however, preferred the gap regions in

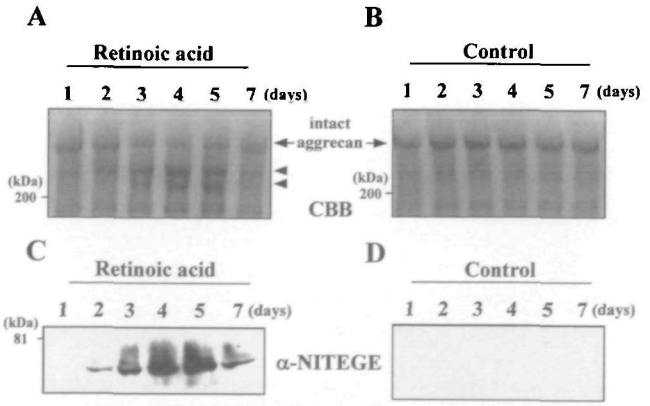


Fig. 1. **Aggrecan-degrading activity of bovine nasal cartilage.** Bovine nasal cartilage was cultured in the presence (A, C) or absence (B, D) of retinoic acid, and the conditioned medium was collected at 24-h intervals. The collected medium was dialyzed against 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, and 5 mM $CaCl_2$, and 10-μl portions of the dialyzed conditioned medium were incubated with 100 μg of purified bovine nasal aggrecan for 10 h at 37°C. After treatment with chondroitinase and keratanase, aggrecan core proteins in the reaction mixtures were analyzed by SDS-PAGE and visualized by staining with CBB (A, B) or by Western blotting with I19C antiserum (C, D). The numbers indicate the days after stimulation by retinoic acid. Arrowheads indicate the aggrecan core fragments generated by the degrading activity.

A

Steps	Protein (mg)	Total activity (mg/h)	Specific activity (mg/h/mg protein)	Yield (%)	Purification (-fold)
Medium	1430	543	0.38	—	1
Q sepharose	448	582	1.30	107	3.42
Zn-chelating	21.6	600	27.8	110	73.2
Ether Toyopearl	2.0	434	217	79.9	571
Mono S	0.015	79.5	5,300	14.6	13,900

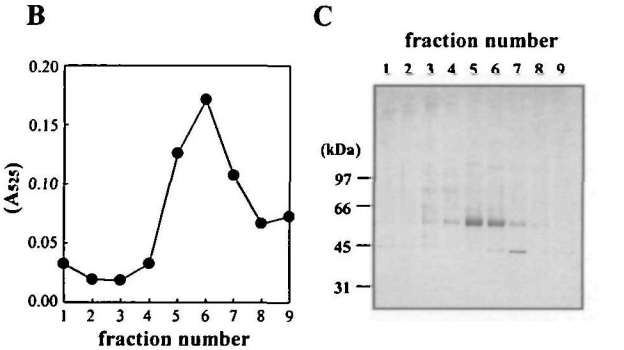
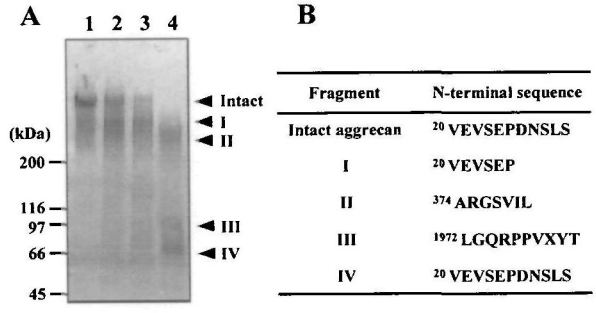


Fig. 2. **Purification of aggrecan-degrading activity in cultured cartilage.** (A) Enzyme activity was determined by SDS-PAGE analysis as described in "MATERIALS AND METHODS." The results are typical of the three experiments carried out. (B) Plot of aggrecan-degrading activity versus fraction number of a Mono S column. The enzyme reactions were carried out in 100 μl of 50 mM Tris-HCl, pH 8.0, containing 5 mM $CaCl_2$, 200 mM NaCl, and 1.2 mg of aggrecan particles. Aggrecanase activity was determined by measuring GAGs released into the solution. (C) SDS-PAGE analysis of the same fractions.

the chondroitin sulfate attachment regions to the aggrecanase site. The enzyme first cleaved the Glu[1971]-Leu[1972] bond in the C-terminal region of aggrecan, generating fragments I (380 kDa) and III (85 kDa). Fragment I was further cleaved at the aggrecanase site into the fragments II and IV (Fig. 3A). Fragment III generated by the first cleavage had the N-terminal sequence of LGQRPPV, which was also found in the medium of interleukin-1 (IL-1)-treated articular cartilage cultures (*6*).

Profiles of Aggrecanase Activity—To test the effect of GAGs on aggrecanase activity, the enzyme was incubated with aggrecan in the presence of various concentrations of heparin and CS. Both heparin and CS inhibited the enzyme in a dose-dependent manner (Fig. 4).



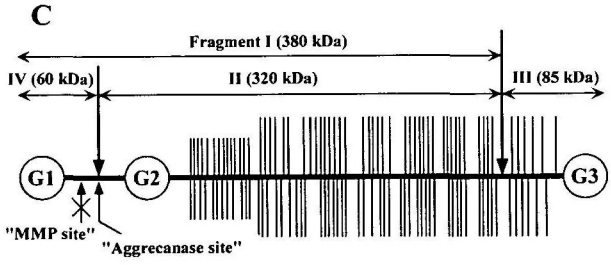


Fig. 3. **Determination of the cleavage sites in aggrecan by aggrecanase.** (A) Aggrecan (2 mg) was incubated with 0 (lane 1), 10 (lane 2), 20 (lane 3), and 100 μl (lane 4) of the Mono S preparation (approximately 2 μg protein/ml) at 37°C for 12 h. After treatment with chondroitinase and keratanase, the reaction mixtures were analyzed by SDS-PAGE and stained with CBB, in which four fragments of aggrecan (I-IV) were detected. (B) N-terminal amino acid sequences of the four fragments produced by the enzyme. (C) Schematic representation of the cleavage sites in bovine aggrecan by the enzyme.

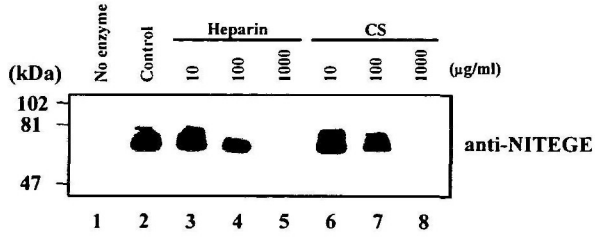


Fig. 4. **Inhibition of aggrecanase activity by heparin and CS.** Aggrecan was incubated with 2 μl of partially purified enzyme preparation (approximately 2 μg protein/ml) and heparin or CS ranging from 0.5 to 50 μg in 50 μl of assay buffer at 37°C for 6 h. After treatment with chondroitinase and keratanase, the reaction mixtures were analyzed by Western blotting with I19C antiserum.

TABLE I. **Effect of protease inhibitors on partial purified aggrecanase.** The inhibitory activity was measured by particle assay. Partially purified aggrecanase was incubated with 1.2 mg of polyacrylamide particles containing aggrecan in the presence of various protease inhibitors at 37°C for 3 h. Aggrecanase activity was determined by measuring the amount of GAGs released from the particles. The concentrations of AG3340, BB94, and CT1746 which gave 50% inhibition of MMP-2 by the assay procedure described previously (*37*) were 4, 0.27, and 0.84 nM, respectively. The data shown are from a single experiment that was repeated twice with very similar results.

Inhibitors	Concentration	Enzyme activity (% of control)
Control	–	100
TIMP-1	1 μM	100
TIMP-2	1 μM	100
EDTA	5 mM	0
EGTA	5 mM	0
Actinonin	10 μg/ml	39
Thiorphan	10 μg/ml	100
Captopril	10 μg/ml	100
1,10-Phenanthroline	10 μg/ml	100
AG3340	660 nM	50
BB94	21 nM	87
	63 nM	50
	524 nM	15
CT-1746	48 nM	50

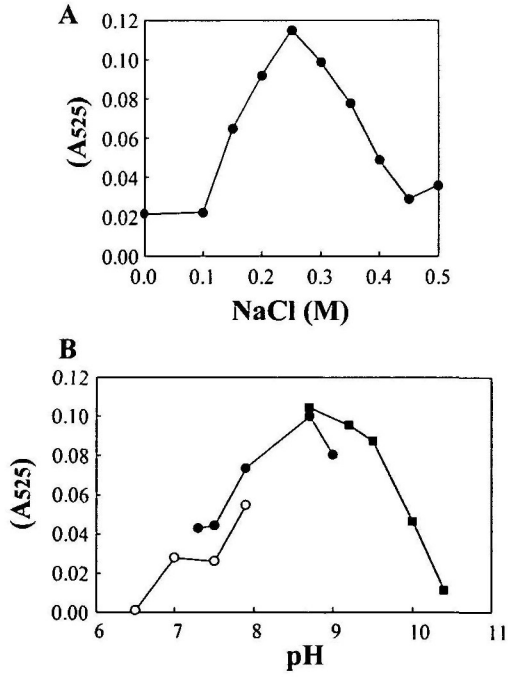


Fig. 5. **NaCl and pH dependency of aggrecanase activity.** (A) The enzyme reactions were carried out in 100 μl of 50 mM Tris-HCl buffer, pH 8.0, containing 5 mM $CaCl_2$, various concentrations of NaCl, and 1.2 mg of aggrecan particles. Aggrecanase activity was determined by measuring GAGs released into the solution. (B) The enzyme reactions were carried out using 1.2 mg of particles in 100 μl of 5 mM $CaCl_2$ and 250 mM NaCl in various buffers (50 mM): for pH 6.5, 7.0, 7.5, and 7.9, MOPS-NaOH (○); for 7.3, 7.5, 7.9, 8.7, and 9.0, Tris-HCl (●); for 8.7, 9.2, 9.5, 10.0, and 10.4, glycine-NaOH (■). Aggrecanase activity was determined by measuring GAGs released into the solution. The data shown are from a single experiment that was repeated twice with very similar results.

Next, the effects of various protease inhibitors were tested (Table I). To obtain quantitative results, the aggrecan particle assay was used instead of Western analysis. The activity was inhibited by EGTA and EDTA, suggesting that aggrecanase is a Ca^{2+}-dependent metalloproteinase. Actinonin slightly inhibited the aggrecanase activity, but most of the common metalloproteinase inhibitors did not affect the activity even at a concentration of 10 μg/ml. On the other hand, the hydroxamate-based MMP inhibitors AG3340, BB94, and CT-1746 inhibited aggrecanase. To determine whether aggrecanase belongs to the MMP family, it was incubated with TIMP-1 or -2, which stoichiometrically inhibit MMPs (*19*). Neither TIMP-1 nor -2 affected aggrecanase activity up to 1 μM. Aggrecanase did not cleave type I, II, or IV collagen, [^{3}H]acetyl gelatin (*20*), or Knight's substrate (*21*) at the enzyme concentrations which cleaved aggrecan completely in a solution assay (data not shown). The same amount of aggrecanase showed no degradation of gelatin under the conditions under which a trace amount of MMP-2 or -9 showed clear negative staining in gelatin zymography (*22*), and showed no degradation of casein in zymography (*23*), in which MMP-3 also showed negative staining (data not shown).

The enzyme had a unique salt-dependency, and the optimal salt concentration was 250 mM (Fig. 5A). The activity displayed a broad pH optimum in the alkaline range with optimal activity at pH 8.0–9.5 (Fig. 5B).

DISCUSSION

While many reports have suggested that a putative aggrecanase is a key enzyme in the destruction of articular cartilage and the loss of cartilage function in patients with joint diseases, the enzyme has remained unknown (*10, 24, 25*). In this study, we have identified and partially purified an aggrecan-degrading activity fulfilling the criteria for aggrecanase. The partially purified enzyme cleaved aggrecan at two sites, both of which were found in the medium of IL-1-treated bovine articular cartilage cultures (*6*).

MMPs are also candidates for the degradation of cartilage under pathophysiological conditions. MMP expression appears to be elevated in joint tissues from patients with OA and RA, and inflammatory cytokines upregulate the expression of these enzymes in both synoviocytes and chondrocytes (*26, 27*). Under limited conditions, MMPs can cleave at the aggrecanase site (*28, 29*). However, all MMPs examined so far preferentially cleave at the MMP site (Asn^{341}-Phe^{342}) within IGD, and none of them has the restricted specificity of aggrecanase (*3, 13*). The fragments derived from the cleavage by MMPs were not detected in cultured chondrocytes stimulated with retinoic acid or inflammatory cytokines (*9*). In the present study, aggrecanase activity was predominantly induced by stimulation of cartilage cultures with retinoic acid, and the partially purified enzyme did not cleave at the MMP site (Fig. 3). Neither TIMP-1 nor -2 inhibited aggrecanase activity at concentrations up to 1 μM (Table I), which would be sufficient to inhibit completely the proteolytic activity of MMPs (*30, 31*). Together with the fact that the enzyme does not cleave type I, II, and IV collagen, it is strongly suggested that aggrecanase belongs to a metalloproteinase family distinct from MMPs.

It is possible that the ionic interaction of the enzyme and GAGs controls the accessibility to the enzyme of the cleavage sites in aggrecan. Aggrecanase preferred the gap region to the aggrecanase site (Fig. 3). This can be explained by the difference in the accessibility of the cleavage sites. The inhibition of the enzyme by heparin and CS (Fig. 4) means that it has binding affinities to heparin and CS. In addition, the enzyme could not cleave the various synthetic peptides corresponding to the aggrecanase site (data not shown). CS has higher molecular weight and more condensed negative charges than KS. These suggest that the enzyme binds to the CS chain first and primarily cleaves the gap region in the CS chains. This hypothesis is further supported by the unique salt-dependency of the enzyme (Fig. 5): the enzyme may bind to CS too tightly for it to cleave the core protein at low salt concentrations, while higher salt concentrations weaken the binding, allowing the enzyme to access the cleavage sites in the core protein more easily.

Aggrecanase is a typical Ca^{2+}-dependent metalloproteinase. Several hydroxamate-based MMP inhibitors inhibited the enzyme, although the concentrations required were 57 to 233 times higher than that for MMP-2 (Table I). This suggests that a similarity in the structure of the active sites in spite of the distinct difference in substrate specificity between aggrecanase and MMPs. These inhibitors have been reported to inhibit another family of metalloproteinases, the reprolysin family (*32*). The reprolysin family is composed of two subfamilies of proteins: the snake venom metalloproteinases (SVMPs) and the mammalian homologues of SVMPs, called a disintegrin and metalloproteinase (ADAM). Atrolysin C, a member of the SVMPs, has the ability to cleave the IGD of aggrecan at both the MMP site and the aggrecanase site (*33*), and three of the ADAM family proteins are expressed in chondrocytes (*34*). Recently, a new type of ADAM family protein, a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS-1), was identified (*35*). Unlike other ADAMs, this protein lacks a transmembrane domain and possesses a thrombospondin (TSP) homologous domain containing TSP type I motif, which is functional for binding to heparin. Aggrecanase is also a secreted protein and has binding affinities to heparin and CS (Fig. 4). Although cleavage of aggrecan by ADAMTS-1 has not been reported to date, aggrecanase might be a homologue of ADAMTS-1.

More recently, Arner *et al.* reported aggrecanase activity in conditioned medium of explant culture (*36*). Their enzyme shares some characteristics with the aggrecanase studied here: both enzymes are induced by retinoic acid or IL-1, cleave the aggrecanase site with an alkaline pH optimum, and have a unique salt-dependency. However, they reported that their enzyme was completely inhibited by TIMP-1 at 500 nM and did not mention other cleavage sites in the gap region between the CS attachment sites. To determine the identity of both these enzymes and to elucidate the pathophysiological functions of aggrecanase(s), further characterization is necessary using both purified and cloned enzymes.

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