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Matrix metalloproteinase-9 in a unique proteoglycan form in avian embryonic growth plate cartilage

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Abstract

The appearance of a high molecular weight gelatinolytic enzyme (230 kDa) correlated with cartilage collagen loss in chick embryonic tibias cultured with lipopolysaccharide. This 230 kDa enzyme was purified and its activity was measured on synthetic and natural substrates. The enzyme was activated by aminophenylmercuric acetate and inhibited by ethylenediaminetetraacetic acid, phenanthroline, marimastat or tissue inhibitors of metalloproteinases. Amino acid sequences of peptides derived from the purified enzyme showed identity with avian MMP-9. Digestion of the intact enzyme with chondroitinase decreased the size of the molecule to 80 kDa on SDS-PAGE. When chick embryonic tibia cultures were radiolabeled with ³⁵S-sulfate, the radiolabel co-purified with the 230 kDa gelatinase. Chondroitinase treated 230 kDa gelatinase also reacted with specific anti-chondroitin sulfate antibodies and FACE analysis revealed a predominance of chondroitin-4-sulfate. These results demonstrate this avian matrix metalloproteinase contained glycosaminoglycan chains. To our knowledge, this is the first report of a matrix metalloproteinase in a proteoglycan form.

Keywords

Avian; embryonic bone formation; extracellular matrix; gelatinase; matrix metalloproteinase; proteoglycan

1.0 INTRODUCTION

The chick embryonic tibia (CET) has been widely used as a model to study long bone development. The major constituents of the CET at 12 days of embryonic development are cartilage, bone marrow and a bony sheath. In these tibias the cartilage under the bony collar hypertrophies and the hypertrophic chondrocytes produce type X collagen[1]. In avian embryonic tibia there is no distinct columnar arrangement of chondrocytes as seen in mammalian growth plates. However, three morphologically distinct regions, which include round (zone 1), flat (zone 2) and hypertrophic (zone 3) chondrocytes, have been described[2]. This embryonic cartilage is remodeled into bone through the resorption of the

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cartilage and the deposition of a bone matrix. Matrix metalloproteinases (MMPs) play key roles in this resorption process both in development [3-8] and in adult skeletal remodeling[9].

MMPs are neutral extracellular enzymes that orchestrate tissue remodeling processes like endochondral bone formation and degenerative conditions like arthritis, and tumor invasion[10-12]. Chief characteristics of MMPs have been described by Woessner[13]. More than twenty members of the MMP family have been identified[14-16]. See the MERROPS protease database (<http://merops.sanger.ac.uk/>). Other related zinc-dependent metalloendopeptidases which share similar mechanisms of activation and catalytic activity with MMPs include the ADAMTS proteinases that cleave procollagens and a variety of proteoglycans[17, 18].

Based on their substrate specificities MMPs have been broadly divided into subclasses: highly specific collagenases (MMP-1, 8, and 13), gelatinases (MMP-2 and 9), and stromelysins (MMP-3, 10 and 11). Avian gelatinase A [19] and gelatinase B-like [20] enzymes have been cloned and biochemically characterized. A full-length avian membrane type-MMP [21] and a partial sequence of avian MMP-13 [22] also have been cloned. Gene regulation for several avian MMPs in pathological conditions has been reported[23].

In order to study the enzymes that orchestrate embryonic cartilage resorption, an in vitro model of cartilage degradation in 12 day intact CET was developed and characterized. In this model LPS stimulates the complete dissolution of embryonic tibial cartilage during a month in organ culture. The appearance of a high molecular weight gelatinolytic enzyme correlated with the collagen loss on days 14-28 of culture[24-27]. Mikuni-Takagaki and Cheng [5] also observed a high molecular weight matrix metalloproteinase in the region of the hypertrophic chondrocytes adjacent to the marrow cavity of embryonic chick explant cultures. Interestingly, this metalloproteinase activity was resistant to known MMP inhibitors. Other investigators have observed a high molecular weight (>200 kDa) avian gelatinase in monolayer growth plate chondrocyte cultures treated with retinoic acid[28].

In this report, we identified the high molecular weight (230 kDa) gelatinase as an avian MMP-9. The apparent high molecular weight of this avian MMP-9 was due to glycosylation of the enzyme with chondroitin sulfate chains.

2.0 MATERIALS AND METHODS

2.1 Unless otherwise stated all standard reagents and materials used were purchased from Sigma Chemical Co. (St. Louis, MO)

2.2 Cultures and Radiolabeling

Twelve-day embryonic chick tibias were dissected and cultured as described by Cole et al. [24]. LPS (Sigma, L-3012, *E. coli* serotype 0111:B4) was added to the culture media (CM) at 10 µg/ml. Culture media were collected every two days, treated with 1 mM phenylmethylsulfonyl fluoride and stored at -80°C. ³⁵S-sulfate (1250 Ci/mmol, GE Healthcare Bio-Sciences Corp., Piscataway, NJ) was added to the media at 20 µCi/ml from day 8 to day 24 of the chick embryonic tibial cultures.

2.3 Zymography and Fluorography

Gelatinolytic activity in CM was assayed by gelatin substrate gels (zymograms) (8% w/v) using a modified method of Heussen and Dowdle[29], as described by Cole[25]. Radiolabeled macromolecules were subjected to SDS-PAGE using 8% (w/v) gels according to Laemmli [30] followed by fluorography[31-33]. Intensities of the bands on the x-ray

films exposed to the dried gels were recorded using a Fluor-S MultiImager (Biorad, Hercules, CA) and quantitatively analyzed using Quantity One software (Biorad).

2.4 Enzyme purification by Gelatin-Agarose Affinity and DEAE-Sepharose Chromatographies

All the steps in the enzyme purification were performed at 4°C. CM having 230 kDa gelatinolytic activity were pooled, dialyzed overnight in affinity chromatography buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM CaCl₂, 0.05% (v/v) Brij-35 and 0.02% (w/v) NaN₃) and applied to a gelatin-agarose affinity column as previously described[19, 34]. Enzyme activity was eluted using a 0-10% (v/v) DMSO (Thermo Fisher Scientific, Rockford, IL) gradient in the same chromatography buffer. Affinity fractions containing 230 kDa enzyme were pooled and dialyzed in diethylaminoethyl (DEAE) chromatography buffer (50 mM sodium acetate, 150 mM NaCl, 4 M Urea, 0.5% (w/v) CHAPS and 0.02% (w/v) NaN₃, pH 6.0) and applied to a DEAE-Sepharose column[19, 35]. The 230 kDa enzyme was eluted using 0.15–1.0 M NaCl gradient in DEAE chromatography buffer at pH 7.0. The eluted fractions were dialyzed in dialysis buffer (5 mM Tris-HCl, pH 7.5, 1 mM CaCl₂, 0.005% (w/v) NaN₃ and 0.005% (v/v) Brij-35) and analyzed by zymography and fluorography. The purified 230 kDa enzyme was stored as aliquots at –80°C.

2.5 Molecular Weight Determination

Molecular weight of 230 kDa enzyme was estimated using molecular weight markers (GE Healthcare Bio-Sciences Corp.). Molecular weight changes of the 230 kDa enzyme after protease-free chondroitinase ABC treatment were determined by plotting the relative mobilities of the enzyme versus the log molecular weight of marker proteins[36, 37].

2.6 Protein and Glycosaminoglycan (GAG) Assays

The protein content of the purified 230 kDa enzyme was measured using a Micro Bicinchoninic Acid (BCA) protein assay kit (Thermo Fisher Scientific) with a bovine serum albumin (BSA) standard. The GAG content of purified 230 kDa enzyme (75 µl) was determined using a dimethylmethylene blue assay (DMB) colorimetric assay[38].

2.7 Chondroitinase or Hyaluronidase Digestion

230 kDa enzyme was digested with protease-free chondroitinase ABC (40 µU) (Seikagaku Co., Tokyo, Japan) in chondroitinase buffer containing 50 mM Tris, pH 8.0, 30 mM sodium acetate, 0.02% (v/v) Brij-35, 0.02% (w/v) NaN₃ and 0.05% (w/v) protease free BSA at 37°C for 2 h. Digested proteins were separated by SDS-PAGE and detected by Coomassie colloidal blue stain (OWL Separation Systems, Woburn, MA). In other experiments, the 230 kDa enzyme (approximately 0.2 µg) was digested with bovine testicular hyaluronidase (1.5 TRU) or Streptomyces hyaluronidase (5 mU) at 25°C for 2 h followed by zymography.

2.8 Papain Digestion

The ³⁵S-sulfate labeled 230 kDa enzyme was digested with papain (0.02 U) in a buffer containing 0.1 M NaH₂PO₄ 5 mM EDTA, 5 mM cysteine-HCl, pH 6.5 at 37°C for 4 h. Enzyme digests were separated by SDS-PAGE and radiolabeled molecules were detected by fluorography.

2.9 Fluorophore-Assisted Carbohydrate Electrophoresis (FACE)

FACE was used to determine the sulfate composition pattern of the GAG chains on the 230 kDa gelatinase as described[39-41]. Briefly, the 230 kDa gelatinase was incubated with a sequential series of enzymes including protease K, Streptomyces hyaluronidase, and chondroitinase ABC. The resultant unsaturated disaccharides were conjugated to 2-

aminoacridone for analysis. The conjugated disaccharides were separated by electrophoresis on MONO composition gels (Glyko, Novato, CA) and the fluorescent disaccharide bands visualized using a Fluor-S Imager with ultraviolet settings. Bands were identified by migration relative to the migration of purified unsaturated disaccharides standards.

2.10 Western Blotting

After SDS-PAGE, the 230 kDa or 72 kDa gelatinases were electrophoretically transferred to nitrocellulose (Micron Separations Inc., Westborough, MA)[42]. The blots were blocked with 5% (w/v) nonfat, dry milk in PBS. Different blots were incubated with the following primary antibodies: anti-C4S (2B6, Chemicon International, Temecula, CA), anti-C6S (3B3, Chemicon), anti-avian MMP-2, anti-rat MMP-13 (Neomarkers, Freemont, CA) and anti-human MMP-9 (Neomarkers). The blots were incubated with species specific secondary antibodies conjugated with horseradish peroxidase (Thermo Fisher Scientific). A SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific) was used with x-ray film to detect the bands on the blots.

2.11 Peptide Sequencing

The 230 kDa enzyme (12 μ g) was treated with protease-free chondroitinase ABC (50 μ U) in chondroitinase buffer at 37°C for 2 h. Digested proteins were separated by SDS-PAGE, stained with Coomassie blue and the resulting 80 kDa band was cut from the gel. Peptides were released from the gel pieces with Lys-C or trypsin and subjected to amino acid sequencing as previously described[43].

2.12 Enzyme Assays Using Synthetic Substrate

7-Methoxycoumarin-4-acetyl-P-L-G-L- -(2, 4-dinitrophenylamino)-A-A-R-NH₂ (MCA-P-L-G-L-DPA-A-A-R-NH₂) (Molecular Probes, Eugene, OR) was used as the fluorogenic synthetic substrate for measuring enzyme activity[44-46]. Unless otherwise specified, 0.120 μ g of 230 kDa enzyme with or without treatment by p-aminophenylmercuric acetate (APMA), inhibitors and/or chondroitinase was added to 1.0 μ M substrate in a 2.2 ml volume of dialysis buffer at 25°C. Enzyme activity at each time point was measured as an increase in fluorescence (λ_{ex} 328nm, λ_{em} 393nm) measured with a SPF-500C spectrofluorometer (SLM Instruments Inc., Urbana, IL). The fluorescence of the end product 7-methoxycoumarin-4-acetyl-P-L (Molecular Probes) was used for calculating the amount of substrate converted to product using the molar extinction coefficient of 20,000 cm⁻¹M⁻¹.

Before the addition of substrate, the 230 kDa enzyme was activated by incubation with p-aminophenylmercuric acetate (1mM) at 37°C for 3 h. For some enzyme reactions the 230 kDa enzyme was digested with 0.5 μ U of protease-free chondroitinase ABC in chondroitinase buffer at 37 °C for 2.5 h. For inhibitory studies, APMA activated 230 kDa enzyme was preincubated with one of the following inhibitors: 10 mM EDTA, 1 mM 1,10-phenanthroline, 1 nM-1 μ M marimastat (Sigma), 10 μ l of serum, 25 μ g γ -2- macroglobulin, 500 ng TIMP-1 (Chemicon) or 500 ng TIMP-2 (Chemicon) for 1.5 hours. When applicable, the APMA activation, chondroitinase treatment and incubation with inhibitor were started in this order and performed simultaneously so the total time for all 3 steps was 3 h.

2.13 Enzyme assays using natural substrate gelatin

³H-gelatin was prepared by heating purified ³H-proline labeled type II collagen from avian chondrocyte cultures to 80°C for 10 minutes [47, 48]. For determining gelatinolytic activity, 5 ng of 230 kDa enzyme was activated by incubation with APMA (1mM) at 37°C for 3 hours and was incubated with ³H-gelatin (18,000 CPM, 1.1 μ g) in a buffer containing 25 mM Tris-HCl, pH 7.4, 10 mM CaCl₂, 0.01% (v/v) Brij-35, 0.01% (w/v) NaN₃ and 0.05% (w/

v) protease-free BSA at 30°C for 15 or 30 minutes. Buffer controls were incubated exactly the same without enzyme. Negative substrate controls contained 20 mM EDTA, no enzyme, and were not incubated. Each assay was performed in triplicate. The enzyme reaction products were separated by SDS-PAGE, and analyzed by fluorography and densitometry. Gelatinolytic activity of 230 kDa enzyme was measured as a loss of ³H-gelatin with time. For studying the influence of TIMPs and/or chondroitinase on enzyme activity, the enzyme was subjected to incubation with TIMP-1, TIMP-2 and/or chondroitinase in addition to APMA in a total assay volume of 25 µl.

3.0 RESULTS

3.1 Cultures

Tibias from the day 12 chick embryos were cultured in DMEM in the presence of LPS. Macroscopic degradation of cartilage started around day 14 of the cultures and the macroscopic loss of cartilage was complete by 28 days of culture as described by Cole et al [24, 25]. LPS has been shown to increase MMP production [20, 49]. CM collected from these cultures contained at least two gelatinolytic enzymes evident on gelatin zymograms (Fig. 1). These enzymes include a lower doublet that was evident throughout the culture period and similar to the avian gelatinase A (72 kDa) described by Cole et al. [26] and Craig et al. [19], and a second diffuse gelatinolytic band in the range of 200 to 260 kDa which was obvious in the CM from day 14. Gelatinolytic enzymes are detected on zymograms even without activation (by APMA), because the SDS in the buffers activates MMPs without cleaving their propeptides [50]. We report here the purification and characterization of the 230 kDa gelatinase.

The 230 kDa enzyme appeared as a polydisperse band and chondroitinase treatment of the 230 kDa enzyme caused the bands to become sharp and migrate to a lower position on zymograms (Fig. 3A) (discussed in detail later). These observations suggested that the GAG chains may be associated with the 230 kDa enzyme. To further study this association tibial organ cultures were radiolabeled with ³⁵S-sulfate and the separation of the enzyme was followed during the purification process by zymography and fluorography.

3.2 Purification

The first step in the purification of the 230 kDa gelatinase was affinity chromatography using gelatin-sepharose as described by Overall [34] (Fig. 2A and B). This affinity purification step resulted in the purification of gelatinolytic enzymes from other media components as well as a partial separation of the 230 kDa enzyme which eluted at slightly lower DMSO concentrations compared to the 72 kDa gelatinase.

3.3 A DEAE column was used as the next step in the purification scheme (Fig. 2C and D)

The 72 kDa enzyme did not bind to the column and eluted in fractions 22-27. However the 230 kDa enzyme did bind and was eluted with 0.5M NaCl in fractions 32-33. Therefore the DEAE column completely separated the 230 kDa gelatinase from the 72 kDa gelatinase. The ³⁵S-sulfate radiolabeled enzyme co-purified with the position of the 230 kDa enzyme on zymograms and fluorograms through both gelatin affinity (Fig. 2B), and DEAE (Fig. 2D) chromatographic separations.

3.4 Chondroitinase and hyaluronidase digestion

Digestion of 230 kDa enzyme with either chondroitinase ABC (Fig. 3A) or bovine testicular hyaluronidase (Fig. 3A) resulted in the migration of the enzyme to a lower molecular weight on zymograms. However digestion of 230 kDa enzyme with *Streptomyces* hyaluronidase did not alter the migration of the 230 kDa enzyme (data not shown). Chondroitinase ABC

cleaves hyaluronan (HA), chondroitin sulfate (CS), and dermatan sulfate; testicular hyaluronidase cleaves HA, and CS, and *Streptomyces* hyaluronidase specifically cleaves HA only. These results suggest the GAG chains present on the 230 kDa enzyme are most likely CS. Neither chondroitinase nor hyaluronidase treatment affected the size of the 72 kDa enzyme (Fig. 3A) demonstrating that the 72 kDa enzyme was not associated with detectable GAG chains.

3.5 The presence of chondroitin sulfate in the purified 230 kDa gelatinase was confirmed by quantitative FACE analysis (Fig. 3B). The 230 kDa enzyme was sequentially digested with proteinase K, streptomyces hyaluronidase and chondroitinase ABC to generate disaccharides from any glycosaminoglycans present. In Fig. 3B fluorescent derivatives of the liberated unsaturated disaccharides from the 230 kDa gelatinase displayed a ~2:1 ratio of chondroitin-4- to chondroitin-6-sulfate.

To determine the size of the protein part of the enzyme, the 230 kDa enzyme (Fig. 4A, lane 2) was digested with protease-free chondroitinase ABC (Fig. 4A, lane 1) followed by SDS-PAGE and Coomassie colloidal blue staining. The molecular weight of the chondroitinase digested enzyme was 80 kDa (Fig. 4A, lane 3) relative to the protein standards (Fig. 4A, lane M).

3.6 Western Blotting

Chondroitinase treated 230 kDa gelatinase gave positive signals (Fig. 4B) when probed with antibodies 3B3 or 2B6 raised against chondroitin-6-sulfate residues or chondroitin-4-sulfate residues, respectively. A stronger signal with the 2B6 antibody for chondroitin-4-sulfate was consistent with the predominance of chondroitin-4-sulfate associated with the 230 kDa gelatinase measured by quantitative FACE analysis (Fig. 3B). The 3B3 or 2B6 antibodies specifically recognize small residual chondroitin sulfate fragments that remain covalently attached to proteins following chondroitinase digestion. In most cases these antibodies do not recognize native intact chondroitin sulfate chains [51]. Thus, a positive signal for these epitopes suggests that small unsaturated saccharide residues are retained covalently-attached to the 80 kDa form of the gelatinase following chondroitinase treatment. This evidence suggests a covalent attachment of GAG chains to the 230 kDa enzyme and is consistent with the high salt elution of this proteoglycan form of the enzyme from the DEAE column.

In order to identify the 230 kDa enzyme, the intact or chondroitinase ABC treated 230 kDa enzyme was probed with antibodies specific for MMP-2, MMP-9 and MMP-13 (data not shown). An antibody raised against avian 72 kDa (MMP-2) did not react with the 230 kDa enzyme but gave a positive signal with avian 72 kDa enzyme suggesting that 230 kDa enzyme was not related to avian MMP-2. Antibody against human MMP-9 or rat MMP-13 also did not react with avian 230 kDa enzyme. However the possibility that 230 kDa enzyme was an avian homolog of MMP-9 or MMP-13 could not be ruled out with these negative results.

3.7 Papain digestion

In order to estimate the size of the GAG chains on the enzyme, ³⁵S-sulfate labeled 230 kDa enzyme (Fig. 4C, lane 1) was digested with papain and analyzed by SDS-PAGE and fluorography. ³⁵S-Sulfate labeled GAG chains released from the 230 kDa gelatinase by papain digestion appeared on the fluorogram as a polydisperse smear with an average apparent molecular weight of about 70 kDa (Fig. 4C, lane 2) relative to denatured protein standards. The actual size of the glycosaminoglycan chains would be much less than their apparent size on SDS-PAGE compared to denatured protein standards.

3.8 Protein and PG contents

The 230 kDa enzyme demonstrated a protein:GAG ratio of 2.5:1 based on protein and DMB sulfated glycosaminoglycan assays.

3.9 Peptide sequencing

In order to identify the 230 kDa enzyme, internal peptides were generated and sequenced. The resulting peptide sequences shown in table 1 were subject to a homology search. These peptides from 230 kDa enzyme showed identity with an avian MMP-9 like molecule[20].

3.10 Enzyme Assays Using Synthetic Substrate

A synthetic fluorogenic peptide, a MCA-P-L-G-L-DPA-A-A-R-NH₂, was used as a substrate to study the activity of the purified 230 kDa enzyme and the influence of chondroitinase treatment, activators or inhibitors on enzyme activity. Initially the fluorescence of methoxycoumarin in the fluorogenic peptide was quenched by energy transfer to the dinitrophenylamino group[44-46]. Fluorescence increases with the cleavage of Gly-Leu (susceptible to cleavage by MMPs) because fluorescence of methoxycoumarin was no longer quenched. Enzyme activity was measured in terms of fluorescence units and plotted versus time.

V_{max} and K_M values for 230 kDa enzyme could not be determined due to a fluorescence quenching problem at high substrate concentrations. Fluorescence units of product generated did not increase linearly with the increase in substrate concentration when the substrate concentration was greater than 10 μM. At high substrate concentrations, fluorescence of one molecule of methoxycoumarin may be quenched by the presence of dinitrophenyl group in the neighboring molecules. However, the enzyme reaction rate was higher at 10 μM compared to the rate at 1.0 μM concentration of substrate. A substrate concentration of 1.0 μM was used in all assays using the synthetic peptide to minimize the possibility of the self-quenching of the products.

3.10.1 APMA Activation—APMA activates latent MMP molecules through the cysteine switch mechanism significantly increasing their enzyme activity. In preliminary studies, optimal time period for maximal enzyme activation by APMA (1 mM) was found to be about 3 hours at 37°C (data not shown). This is similar to the observations of Hahn-Dantona et al.[20] for an avian 75 kDa gelatinase B-like enzyme. For all subsequent enzyme assays, 3 hour APMA activated 230 kDa enzymes were used. Fig. 5 shows the majority of the purified MMP-9 was in a latent form. APMA activation increased the rate of fluorogenic peptide cleavage by 7-fold.

3.10.2 Chondroitinase Treatment—The 230 kDa enzyme (0.12 μg) was digested by 0.5 μU of chondroitinase ABC at 37°C for 2.5 hours. As analyzed by zymograms, treatment completely removed GAG chains from the 230 kDa enzyme under these conditions. Chondroitinase treatment to remove GAG chains from the enzyme did not alter the 230 kDa enzyme activity on the synthetic substrate (Fig. 5).

3.10.3 Inhibition—The activity of the enzyme was completely inhibited by EDTA and substantially inhibited by phenanthroline (data not shown). After the addition of the enzyme-inhibitor mixture to the substrate solution, the phenanthroline concentration was diluted 50 fold and fell below the required concentration for the effective inhibition of MMPs.

However the phenanthroline concentration could not be increased because it had substantial fluorescence by itself and interfered with the assay. Marimastat, a potent hydroxamate inhibitor of MMPs, showed 50% inhibition of the gelatinase at 10 nM, and complete

inhibition at 100 nM (data not shown). Marimastat has been shown to inhibit MMP-9 in the low nM range[52].

Activity of the 230 kDa enzyme (0.06 μ g) was completely inhibited by 500 ng of TIMP-1 or TIMP-2 (Fig. 6A). But, when the quantity of the TIMPs was 200 ng, the inhibition was only partial (data not shown). Bovine calf serum, fetal calf serum, and γ -macroglobulin were unable to inhibit the MMP-9 enzyme activity using the synthetic peptide assay (data not shown).

Mikuni-Takagaki and Cheng [5] have suggested that the high molecular weight gelatinase is not readily inhibited by TIMPs and serum inhibitors. Therefore one potential function of GAG chains on the 230 kDa gelatinase may be to alter enzyme activity or modify inhibitor binding to the enzyme. Fig. 6B shows chondroitinase ABC treated 230 kDa gelatinase did not change the ability of TIMP-1 or TIMP-2 to inhibit the enzyme when measured with the synthetic peptide substrate.

3.11 Although the GAG chains on this avian MMP-9 did not affect the activation, activity or inhibition of the enzyme with the peptide substrate, the possibility exists that the GAG chains may interfere with the proteolysis of the natural substrate gelatin which is much larger than the synthetic peptide substrate. Therefore 3 H-gelatin derived from denatured, pepsinized type II collagen also was tested as a substrate for the enzyme. Digestion of the 3 H-gelatin molecule was followed by SDS-PAGE and fluorography. Densitometric analysis of fluorograms from these enzyme assays using radiolabeled gelatin yielded the data reported in Table 2. About half of the radiolabeled gelatin substrate was degraded by the enzyme in 30 minutes. The enzyme activated with APMA degraded 1.03 μ g of type II gelatin / μ g enzyme /minute at 30°C. The purified MMP-9 showed similar activity toward this substrate independent of the association of GAG chains with the enzyme. In the presence of TIMP-1 or TIMP-2 most of this enzyme activity was inhibited. The data show that when the GAG chains were removed from the 230 kDa gelatinase, the inhibition of the enzyme activity on the natural gelatin substrate by either TIMP-1 or TIMP-2 was not affected. Therefore chondroitinase ABC treatment did not alter the MMP-9 enzyme activity or its inhibition by TIMPS.

4.0 DISCUSSION

A high molecular weight enzyme with gelatinolytic activity has been observed in embryonic [5, 25, 26] and post-hatch [28] avian cartilage. This enzyme was purified from the medium of embryonic tibial explant cultures treated with LPS. The enzyme was purified in two steps by chromatography on gelatin- and DEAE-sepharose. Amino acid sequencing of Lys-C generated peptides from the molecule showed it to be highly homologous to avian MMP-9, the 75 kDa gelatinase B-like enzyme, cloned by Hahn-Dantona et al.[20].

The purified enzyme displayed a polydisperse size on SDS-PAGE and gelatin zymograms with an average of about 230 kDa. This size is much greater than the 75 kDa size reported for the enzyme derived from the avian macrophage cell line HD11 and from avian embryonic bone marrow cells at 18-19 days of development[20]. This size is similar to that reported for a high molecular weight (>200 kDa) gelatinase from 1 day post-hatch avian epiphyseal growth plate cartilage[28]. Those investigators suggested this size was due to a complex of two 75 kDa gelatinase B dimers with two lipocalin 25 kDa dimers. However the results reported here demonstrate the high molecular weight of the 230 kDa gelatinase from embryonic cartilage explant cultures is due to the covalent attachment of GAG chains to the enzyme making it a proteoglycan.

This avian 230 kDa gelatinase was digested by chondroitinase ABC and testicular hyaluronidase to a size of 80 kDa. FACE analysis of the chondroitinase digest revealed a combination of chondroitin 4- and chondroitin 6-sulfate disaccharides in a 2:1 ratio were present. This is consistent with the fact that antibodies specific for chondroitin 4- and chondroitin 6-sulfate detected these residues on the chondroitinase digested stubs remaining on the chondroitinase digested form of the 80 kDa molecule on Western blots. Interestingly, chondroitin-6-sulfate is the predominant disaccharide component of large proteoglycans (e.g., aggrecan) present within cartilage of 12-day CET[53, 54]. This would suggest that the chondroitin sulfate associated with the 230 kDa gelatinase is not due to a contaminating or cross-linked proteoglycan. Papain digestion of the ³⁵S-sulfate labeled MMP-9 released chondroitin sulfate chains with an average mobility comparable to denatured 70 kDa protein standards by SDS-PAGE.

The association of chondroitin sulfate chains with MMP-9 appeared to be covalent. The ³⁵S-sulfate radiolabel co-purified with the enzyme during elutions with DMSO from the gelatin column and in 4 M urea on the DEAE column. The enzyme eluted from the DEAE column in 0.5 M NaCl as would be expected for a proteoglycan. The 230 kDa size of the enzyme also did not change after ultracentrifugation under dissociative conditions in 4 M CsCl and 4 M GuHCl (data not shown, most enzyme activity was lost). Unless the GAG chains were covalently attached to the protein, the 230 kDa enzyme would not remain intact in 4 M urea, 0.5 M NaCl, 4 M CsCl or 4 M GuHCl. The anti-chondroitin sulfate antibodies 2B6 and 3B3 recognized residual unsaturated chondroitin sulfate stubs on the 80 kDa form of the gelatinase after chondroitinase treatment. These data demonstrate that avian embryonic growth plate MMP-9 can exist in a proteoglycan form. The deduced amino acid sequence of the avian gelatinase B-like enzyme contains six -S-G- sequences and three -G-S- sequences which may represent putative O-linked chondroitin sulfate chain attachment sites. A few of these sites are flanked by acidic amino acids, but none display the consensus sequence -D/E-X-S-G-X- typical of GAG chain attachment sites on many proteoglycans[55]. Of the 9 putative GAG attachment sites only one of these sequences is conserved in human MMP-9 which does not contain GAG chains.

Does the presence of CS chains on MMP-9 alter enzyme activity or interfere with MMP-9 inhibitors? The observation that this 230 kDa gelatinase is a PG with covalently attached CS chains is unique for a MMP. It offers the opportunity to test whether GAG chains affect the function of this enzyme. A synthetic peptide with quenched fluorescence was used to evaluate the activity of the enzyme. The ability of the GAG chains to affect the enzyme assays was tested by removing the CS chains from the enzyme with protease-free chondroitinase ABC. The data showed that the removal of CS chains from the 230 kDa gelatinase did not affect the activity of the enzyme.

The activity of MMPs *in vivo* also is controlled by groups of inhibitors which include the TIMPs and α_2 -macroglobulin. These molecules bind stoichiometrically to the MMPs and inactivate the enzymes. The GAG chains might interfere with the binding of these inhibitors. Mikuni-Takagaki and Cheng [5] may have been working with a truncated form of this same enzyme and made the observation that this gelatinase was not inhibited by serum, α_2 -macroglobulin or TIMPs. If this were true it would be an extremely important observation, because most MMPs are inactivated by these inhibitors. This would suggest that once the enzyme was activated it is difficult to inactivate *in vivo*. Van den Steen et al. have suggested that removal of sialic acid from human neutrophil MMP-9 decreases the inhibitory effect of TIMP-1 binding[56, 57]. An O-glycosylated domain, located upstream of the hemopexin domain, of MMP-9 is required to correctly orient the hemopexin domain for inhibition by TIMP-1[57]. Therefore it is possible that the GAG chains on the 230 kDa enzyme also may interfere with the binding of these inhibitors. Several experiments were performed to test if

the presence of GAG chains affected the inhibition of the 230 kDa enzyme by several MMP inhibitors.

High concentrations of TIMP-1 and TIMP-2 were needed to inhibit the 230 kDa gelatinase, which might explain why Mikuni-Takagaki and Cheng [5] did not detect inhibition by TIMPs. Bovine calf serum, fetal calf serum or α_2 -macroglobulin did not inhibit enzyme activity in assays using synthetic peptide and removal of GAG chains did not make any difference. α_2 -macroglobulins are large glycoproteins found in serum. They are widely considered as inhibitors of proteases [58] including mammalian collagenases. Binding of a proteinase to the 'bait' region of α_2 -macroglobulin leads to cleavage and large conformational changes in α_2 -macroglobulin thereby trapping the proteinase (MMP). However using a synthetic peptide substrate, the α_2 -macroglobulin does not necessarily inhibit the activity of MMP. Hori and Nagai [59] showed that collagenase complexed with α_2 -macroglobulin has still an unmasked active site to which small synthetic peptides have access and were cleaved.

Under our experimental conditions using a synthetic peptide or radiolabeled gelatin derived from type II collagen, removal of GAG chains by treating the 230 kDa enzyme with chondroitinase ABC did not show any significant difference in the activity or inhibition compared to the intact 230 kDa enzyme. However, the behavior of this enzyme *in vivo* may be more complex and the GAG chains on the 230 kDa enzyme may have other functions.

This is the first report of a MMP, specifically MMP-9, existing in a proteoglycan form. Multiple lines of evidence a) co-purification of the ^{35}S -sulfate radiolabel with enzyme activity, b) high salt elution from a DEAE column, c) enzyme digestions, d) Western blotting with anti-CS antibodies, and d) FACE analysis support this conclusion. A number of other macromolecules exist as part-time proteoglycans. Examples include type IX collagen[60] and the mouse Ia associated invariant chain[61]. Glycosaminoglycan modifications on proteins may or may not alter their function. The presence of a GAG chain on betaglycan is necessary for inhibition of TGF-beta signaling in renal epithelial LLC-PK1 cells[62]. We were unable to detect changes in gelatinolytic activity of this avian MMP-9 or its inhibition either with or without the GAG chains. However it is reasonable to expect the high negative charge of the GAG chains on avian MMP-9 in embryonic cartilage may facilitate binding to basic proteins like collagen. A recent observation that suggests proteoglycan-collagen interactions are critical to connective tissue integrity is the fact that single nucleotide mutations in Osteogenesis Imperfecta patients which convert glycine residues to another amino acid have a tendency to be lethal if they cluster in regions of the 2(I) chain where type I collagen binds proteoglycan molecules[63]. Future studies will determine if the CS chains on this MMP-9 proteoglycan facilitate interactions with other macromolecules in avian growth plate cartilage.

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ABBREVIATIONS

APMA	p-aminophenylmercuric acetate
CET	chick embryonic tibia
CM	conditioned medium

CS	chondroitin sulfate
C4S	chondroitin-4-sulfate
C6S	chondroitin-6-sulfate
DMSO	dimethylsulfoxide
ECM	extracellular matrix
FACE	fluorophore-assisted carbohydrate electrophoresis
GAG	glycosaminoglycan
HS	heparan sulfate
LPS	lipopolysaccharide
MMP	matrix metalloproteinase
PG	proteoglycan
TIMP	tissue inhibitor of metalloproteinase

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HIGHLIGHTS

- Lipopolysaccharide induced a 230 kDa gelatinase in cultured chick embryonic tibias
- The gelatinase was purified by gelatin affinity and DEAE chromatography
- $^{35}\text{SO}_4$ -labeling and chondroitinase treatment showed the enzyme contained CS chains
- Removal of the GAG chains did not affect enzyme activity or inhibitor binding
- Sequencing showed the enzyme to be avian MMP-9 in a proteoglycan form

Figure 1. Gelatin zymograms of 2 μ l of CM from LPS treated chick embryonic tibial organ cultures. Gelatinolytic activity was detected as cleared bands. A low molecular weight doublet (~72 kDa avian gelatinase) was observed throughout the culture period and high molecular weight (~230 kDa) gelatinolytic activity was prominent on days 14 to 28.

Figure 2.

Purification of gelatinolytic enzymes on a gelatin-sepharose (frames A and B) and DEAE-sepharose (frames C and D) columns. Frames A and C show zymograms of gelatinolytic enzymes and frames B and D show fluorograms of ^{35}S -sulfate labeled molecules. Lane M contained pooled media from tibial organ cultures. Affinity purification (frames A and B) fractions 1-9 were unbound fractions. Fractions 10-19 were bound fractions eluted with a linear 1-10% DMSO gradient. The 230 kDa enzyme eluted first followed by an overlap with the 72 kDa enzyme. Pooled gelatinolytic enzyme fractions (lane A) from the gelatin-sepharose column were applied to the DEAE column (frames C and D). Fractions 21-27 were unbound fractions. Fractions 28-34 were eluted with a linear 0.15-1.0 M NaCl gradient. The 72 kDa enzyme in fractions 22-27 did not bind to the column and was not labeled with ^{35}S -sulfate (frame D). The 230 kDa enzyme bound to DEAE-Sepharose and was eluted with 0.5 M NaCl in fractions 32 and 33. ^{35}S -sulfate label co-purified with 230 kDa enzyme. Other gelatinolytic enzymes present in fractions 28-30 were separated from the 230 kDa gelatinase, but were not further characterized.

Figure 3.

Frame A: Zymograms showing chondroitinase ABC and testicular hyaluronidase digestions of the 230 and 72 kDa gelatinases. After digestion with either enzyme, 230 kDa gelatinase band showed a mobility shift, whereas no shift was observed for the 72 kDa gelatinase band. Frame B: FACE analysis of purified 230 kDa gelatinase showing the unsaturated disaccharide content of C4S and C6S (lane 2) compared to unsaturated disaccharide standards (lane 1).

Figure 4.

Macromolecular composition of the 230 kDa gelatinase. Frame A shows a chondroitinase ABC digestion of the 230 kDa gelatinase on SDS-PAGE stained with Coomassie colloidal blue stain. Lanes M, molecular weight standards; 1, chondroitinase ABC enzyme alone; 2, the 230 kDa gelatinase; and 3, chondroitinase ABC digest of the 230 kDa gelatinase. A greater quantity of chondroitinase (1 μ g) was added in lane 1 to be able to identify the position of the enzyme compared to lane 3. Frame B: Western blot detection of unsaturated CS oligosaccharide fragments on the gelatinase. Both blots detected an 80 kDa band after incubations with either a C4S specific 2B6 antibody or a C6S specific 3B3 antibody. Frame C shows a fluorogram of 35 S-sulfate labeled 230 kDa gelatinase before (lane 1) and after (lane 2) papain digestion. In lane 1 the upper band is assumed to be an aggregate of 230 kDa gelatinase.

Figure 5.

MMP enzyme assay using peptide substrate. The effects of APMA activation and chondroitinase ABC treatment on the enzyme activity of the 230 kDa gelatinase were tested using a quenched fluorescent synthetic peptide substrate. APMA activated the latent enzyme and increased the activity of the 230 kDa enzyme about 7 fold. Chondroitinase treatment had no effect on this activity. The following symbols represent kinetics for reactions with substrate alone (open circles), substrate plus enzyme (open triangles), APMA activated enzyme (open diamonds), and activated enzyme after chondroitinase treatment (open squares).

Figure 6.

Influence of TIMPs and chondroitinase ABC treatment on 230 kDa gelatinase activity. In all assays the 230 kDa gelatinase was APMA activated and incubated with the peptide substrate in presence of 500 ng of TIMP-1 or TIMP-2 either without (frame A) or after chondroitinase treatment (frame B). Both TIMP-1 and TIMP-2 almost completely inhibited 230 kDa gelatinase activity. Chondroitinase treatment had no effect on the ability of the TIMPs to inhibit the enzyme. In frames A and B the closed diamonds represent enzyme reactions with no inhibitor, with open triangles for TIMP-1, or with open circles for TIMP-2.

Table 1

Sequenced peptides from the 230 kDagelatinase

Sequenced Peptides	Homology (%) ^I
VLLFSGEHWRLDVK	100
DSFYWR	100
YDLLQCPQH	100
GYPRDTDDVFTGVPLDAR	100
AVIDAAFQDVL	100
SGTQGAF <u>L</u> IADTWPGLP	94
FDAITEIT <u>G</u> ELHFFK	93

^I Percent homology with chicken gelatinase B like enzyme reported by Hahn-Dantona et al. [20]. Underlined residues differed from those in data base.

Table 2³H-gelatin digestion by the 230 kDa gelatinase¹

Samples	30 min
³ H-gelatin (control)	0 +/- 3.7
³ H-gelatin + 230 kDa	57.8 +/- 1.2
³ H-gelatin + 230 kDa + Chondroitinase	59.8 +/- 4.8
³ H-gelatin + 230 kDa + TIMP-1	15.8 +/- 4.5
³ H-gelatin + 230 kDa + TIMP-1 + Chondroitinase	11.9 +/- 0.7
³ H-gelatin + 230 kDa + TIMP-2	15.7 +/- 5.5
³ H-gelatin + 230 kDa + TIMP-2 + Chondroitinase	10.1 +/- 3.7

¹Percent of optical density units lost from ³H-gelatin control +/- standard deviation in triplicate assays. EDTA control and buffered control were identical. All enzyme samples were activated with APMA.