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Extracellular matrix of ostrich articular cartilage

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ABSTRACT: The composition and organization of the extracellular matrix of ostrich articular cartilage was investigated, using samples from the proximal and distal surfaces of the tarsometatarsus. For morphological analysis, sections were stained with toluidine blue and analyzed by polarized light microscopy. For biochemical analysis, extracellular matrix components were extracted with 4 M guanidinium chloride, fractionated on DEAE-Sephacel and analyzed by SDS-PAGE. Glycosaminoglycans were analyzed by electrophoresis in agarose gels. Structural analysis showed that the fibrils were arranged in different directions, especially on the distal surface. The protein and glycosaminoglycan contents of this region were higher than in the other regions. SDS-PAGE showed the presence of proteins with molecular masses ranging from 17 to 121 kDa and polydisperse components of 67, 80-100, and 250-300 kDa in all regions. The analysis of glycosaminoglycans in agarose-propylene diamine gels revealed the presence of only chondroitin-sulfate. The electrophoretic band corresponding to putative decorin was a small proteoglycan containing chondroitin-sufate and not dermatan-sulfate, unlike other cartilages. The higher amounts of proteins and glycosaminoglycans and the multidirectional arrangement of fibrils seen in the distal region may be correlated with the higher compression normally exerted on this region.

Introduction

The mechanical properties of articular cartilage and other connective tissues are determined by the composition of the extracellular matrix (ECM) and by interactions between its components (Blaschke *et al*., 1996). Articular cartilage facilitates the sliding between the articular surfaces and absorbs the shock of mechanical impacts.

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Collagens and proteoglycans (PGs) are the most abundant components of the cartilage matrix (Iozzo *et al.,* 1994; Eyre, 2002), although there are also non-collagenous glycoproteins (Neame *et al.,* 1999; Roughley, 2001). Cartilage generally contains collagen of types I, II, V, VI, IX and XI (Van der Rest and Garrone, 1991; Vanwanseele *et al.,* 2002). Type II collagen predominates in articular cartilage (Eyre, 2002), and forms basic fibrillar structures that provide resistance to tension (Schimidt *et al.,* 1990; Reichenberg and Olsen, 1996). Type IX (Reichenberg and Olsen, 1996) and type XI (Sandberg *et al.,* 1993) collagen are associated with type II collagen. The collagen fibers are normally embedded into a hydrated gel of PGs and other glycoproteins (Blaschke *et al.,* 1996).

The predominant glycosaminoglycan (GAG) of PGs in articular cartilage is chondroitin-sulfate (CS)

(Knudson and Knudson, 2001), with aggrecan being the most abundant CS proteoglycan in the matrix of articular cartilage (Kiani *et al.,* 2002). Small PGs such as decorin, biglycan and fibromodulin may also be present (Hocking *et al.,* 1998). Decorin and fibromodulin are associated with collagen fibrils (Knudson and Knudson, 2001), and probably have a role in organizing collagen fibrils and in maintaning tissue integrity (Hocking *et al.,* 1998).

Structurally, articular cartilage is divided into four zones: superficial, middle, deep and calcified zones, with the latter representing a transition between articular cartilage and bone (Ratcliffe and Mow, 1996). Although that the ECM of different vertebrates has a similar basic composition, in some species matrix varies in the composition and organization, depending on the body weight, posture and physical activity. The ostrich *(Struthio camelus)* is the largest living bird species, and has peculiar anatomical and functional characteristics. These birds are increasingly being used as an alternative source of meat but develop arthrosis when reared in small enclosures. However, nothing is known about the composition and the structure of the ECM of articular cartilage in the tarsometatarsus of these birds. In this work, we investigated the characteristics of the ECM of ostrich articular cartilage and identified the main components and their organization to resist tension and compression of the joints.

Material and Methods

Twenty-four-month-old ostriches of the *African Black* lineage, were used. The lateral (PL) and the medial (PM) portions of the proximal articular cartilage and the central (DC) part of the distal articular cartilage of the tarsometatarsus, were obtained from 15 animals (5 for morphology and 10 for biochemical procedures) immediately after slaughter in a commercial slaughterhouse. The fragments were placed in fixative solution for subsequent morphological analysis, or frozen at -20°C for biochemical analysis. Healthy articular cartilage had a white color and a smooth crystalline aspect.

Fixation and staining

The cartilage fragments were fixed in 4% paraformaldehyde in Millonig's buffer, pH 7.4, for 24 h, and then processed for routine inclusion in paraffin Histosec/Paraplast Plus (Merck), Sections of 7 μm thick were cut tangentially to the articular surface and stained with 0.025% toluidine blue solution in McIlvaine buffer, pH 4.0 (Mello and de Campos Vidal, 1980).

Polarization microscopy

The organization of the ECM was analyzed with Zeiss Axioplan 2 microscope, adapted for qualitative analyses of optical anisotropy and loaded with Kodak Gold ISO 100 film. To acces birefringence, the polarizer was crossed and the material was positioned at a 45° angle relative to the polarizer.

Extraction procedures

Slices of the PL, PM and DC regions were homogenized in 12 volumes of PBS (0.15 M NaCl, 5 mM Na_2HPO_4, 50 mM EDTA, 1 mM PMSF, pH 7.4), with a Brinkman PT 1200C homogenizer. After centrifugation (39,000 x g, 4°C, 50 min) the precipitate was treated with 15 volumes of 4 M guanidine chloride (GuHCl), containing 1 mM PMSF and 20 mM EDTA in 50 mM sodium acetate buffer, pH 5.8 (Heinergård and Sommarin, 1987) at 4°C for 24 h. The mixture was then centrifuged (39,000 x g, 4°C, 50 min) and the supernatant used for biochemical analysis.

Quantitative analysis

Protein was quantified according to the method of Bradford (1976), using bovine serum albumin as standard. Sulfated GAGs were quantified by the dimethylmethylene blue method (DMMB) (Farndale *et al.,* 1986) using CS as standard. To quantify hydroxyproline, fragments of the three cartilage regions were hydrolyzed in 6 N HCl (1 mL/10 mg tissue of wet) for 4 h at 130°C. The hydrolysate was then treated with cloramine T solution and perchloric acid/aldehyde, as described by Stegemann and Stalder (1967). After incubation for 15 min at 60°C, the absorbance was read in a spectrophotometer at 550 nm.

Chromatography

The GuHCl extracts of each region were dialyzed against 7 M urea in 50 mM Tris-acetate buffer, pH 8.0, and the components separated by chromatography on a column (1.5 x 2.7 cm) of DEAE-Sephacel previously equilibrated with 7 M urea, as described above. The proteins and proteoglycans were eluted at a flow rate of 1.5 mL/min using a gradient of 0.1 - 1.0 M NaCl in buffered 7 M urea. Fractions (2.8 mL) were collected

FIGURE 1. Tangential sections of ostrich articular cartilage stained with toluidine blue and viewed with non-polarized (A,C,E) and polarized (B,D,F) light microscopy. A,B - PL region showing randomly associated metachromatic material (*) associated with fibrils. Some regions with fibrillar material, are weakly stained (►) (A). Some sections positioned at 45° relative to the polarizers, showed fibrils in different directions (→), although most were arranged in the antero-posterior direction (B). C,D - PM region, with a morphology similar to the PL region, except that the fibrils are much more compacted and show a more organized distribution, as may be seen from the birefringence image (D). E,F - DC region showing a large amount of metachromatic material (*) and fibers running in several directions (→) X 40.

and the elution profile was monitored spectrophotometrically based on the absorbance at 280 nm (Hewlett Packard 8452 A).

Sodium dodecyl sulfate- electrophoresis (SDS-PAGE)

SDS-PAGE was done according to Zingales (1984) using gradient gels (4-16%). Samples of the chromatographic fractions were precipitated in acetate-ethanol (Heinegård and Somarin, 1987) and analyzed by SDS-PAGE. Staining was done with Coomassie brilliant blue R-250. The relative molecular masses were deduced from the Rf of molecular mass markers (Weber and Osborn, 1969).

β-elimination

To obtain free GAGs from the PGs eluted from the DEAE-Sephacel column, samples were precipitaded with acetate-ethanol and incubated for 19 h with 0.5 M NaOH at 4°C, precipitated with ethanol and washed with acetone (Michelacci and Horton, 1989). The GAGs were analyzed in agarose gels.

Enzymatic treatments

Digestion with papain

To extract GAGs from the tissue, cartilage fragments were incubated in acetone overnight at 4°C, dried at 37°C, and treated with papain (40 mg/g tissue) in 0.03 M sodium citrate buffer, pH 3.5, containing 0.04 M EDTA and 0.08 M β-mercaptoethanol (Michelacci and Horton, 1989). The GAGs recovered after precipitation with ethanol were analyzed in agarose gels.

Digestion with chondroitinase ABC/AC

Chondroitinases ABC and AC purchased from Seikagaku were used for the digestion. After β-elimination, the sample was suspended in 10 µL of sodium acetate buffer, 50 mM Tris and 10 mM EDTA, pH 8.0 for chondroitinase ABC and pH 6.0 for chondroitinase AC (Beeley, 1985). The proportions used were 0.03 U of chondroitinase ABC and 0.07 U of chondroitinase AC per 100 µg of GAG, as determined with DMMB. After 20 h at 37°C, the GAGs were precipitated in ethanol and analyzed in agarose-propylene diamine gels.

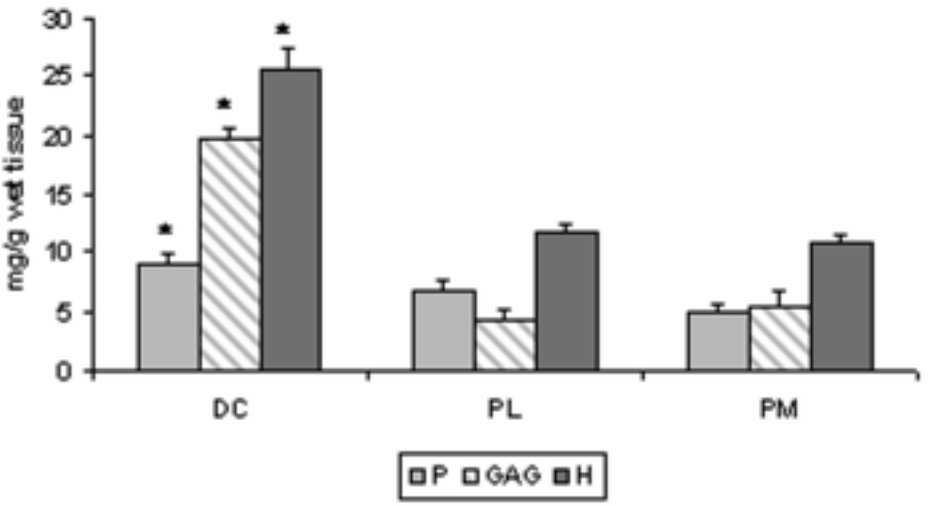

FIGURE 2. Protein (P), glycosaminoglycan (GAG) and hydroxyproline (H) contents of the DC, PL and PM regions. Greater amounts of the three compounds were present in the DC region. There were no differences between PL and PM the columns on the mean ± S.E.M. of five determinations. * $p < 0.05$ vs the corresponding column in PL and PM (ANOVA).

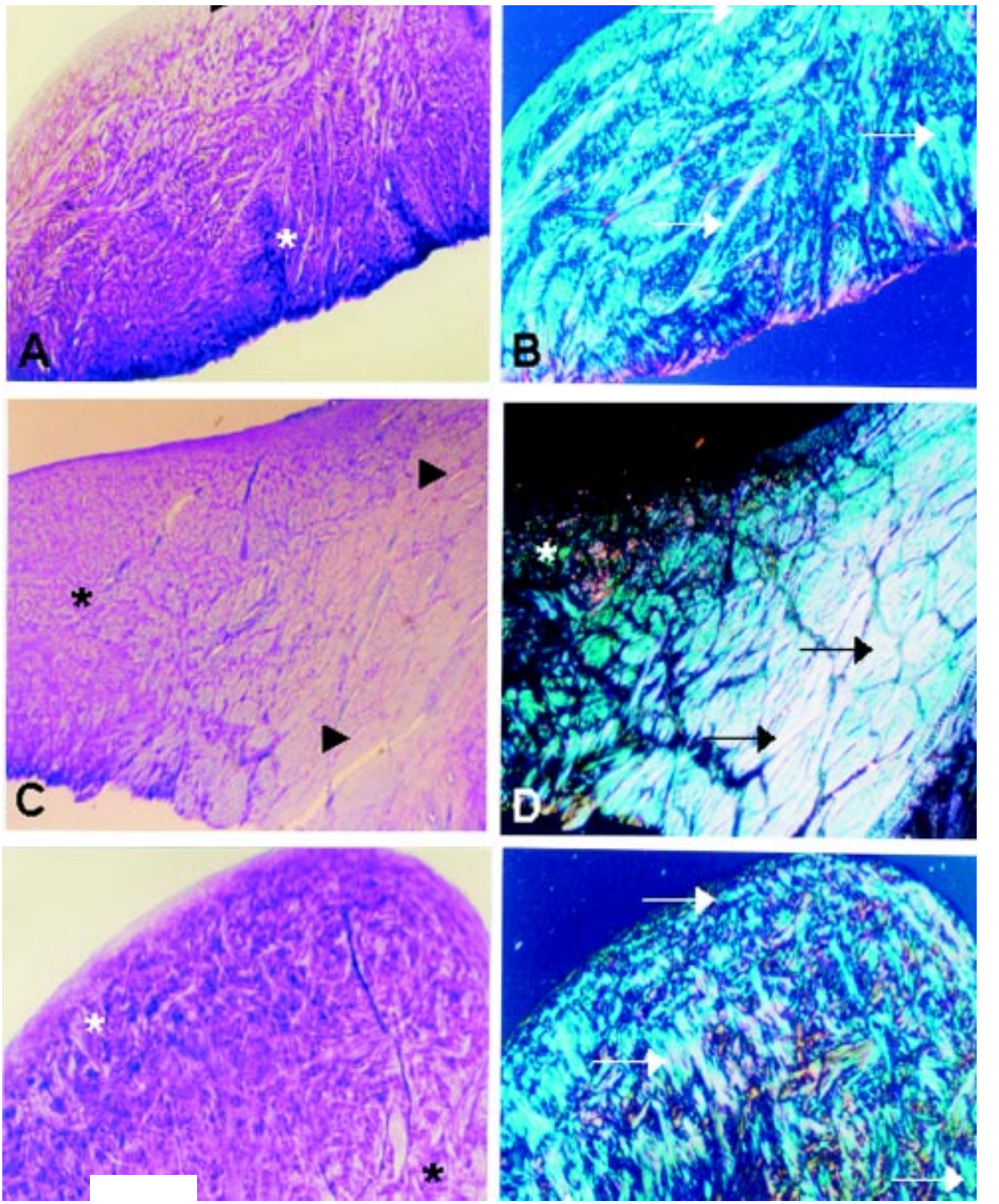

FIGURE 3. DEAE-Sephacel chromatography (A,C,E) and SDS-PAGE (B,D,F) of PL, PM and DC extracts. The proteins were eluted with a gradient (0.1-1.0 M) of NaCl. **G** - start of the gradient. The gels show proteins with molecular masses 121, 61, 47, 36, 21 and 17 kDa. Polydisperse bands were also seen at 67, 80-100 and 250-300 kDa (▶).

Electrophoresis in agarose gels

The GAGs obtained by enzymatic digestion were analyzed by electrophoresis in agarose gels in 0.05 M 1.3-diaminopropane-acetate buffer, pH 9.0 (PDA), as described by Dietrich and Dietrich (1976), using CS, dermatan-sulfate (DS) and heparan-sulfate (HS) as standards.

Statistical analysis

The results were expressed as the mean ± S.E.M., when appropiate. Statistical comparisons were done using analysis of variance One-Way ANOVA (Unstacked), with a value of $p < 0.05$ indicating significance.

Results

Morphology

Examination of toluidine blue stained sections of the PL, PM and DC regions by normal light microscopy (Fig. 1A,C,E) showed fibrous components corresponding to collagen bundles and metachromatic material. The DC region showed strong metachromasy because of the ordered binding of toluidine blue molecules to polyanionic molecules associated along the collagen bundles (Fig. 1E). The PL (Fig. 1A) and PM (Fig. 1C) regions, particularly the latter, showed a non-uniform distribution of the metachromatic regions, indicating that the anionic components of the ECM were arranged in a heterogeneous manner. Analysis of these same sections using polarized light microscopy (Fig. 1B,D,F) showed fibers with marked birefringence, suggesting that they were organized and oriented preferentially parallel to the antero-posterior direction of the proximal and distal cartilage of the tarsometatarsus. In addition to these fibers, there were fibrils arranged obliquely to the antero-posterior axis, especially in the DC region which is subject to a larger compressive load, in relation to the PL and PM regions.

Biochemistry

The protein and sulfated GAGs concentration of the GuHCl extracts, and hydroxyproline of the tissue, of the three regions are shown in figure 2. Higher concentrations of proteins, hydroxyproline and GAGs were found in the DC region compared to PL and PM. There were no significant differences in the levels of the components between PL and PM.

DEAE-Sephacel chromatography of GuHCl extracts from the three regions (Fig. 3A,C,E), revelead the presence of cationic and anionic components. The components that eluted with the NaCl gradient were analyzed by SDS-PAGE. In all regions proteins with molecular masses 121, 61, 47, 36, 21 and 17 kDa were found (Fig. 3B,D,F), as were polydisperse bands of 67, 80-100 and 250-300 kDa. These diffused bands may correspond to the small PGs fibromodulin, decorin and biglycan, respectively.

The analysis of GAGs in agarose gels after digestion of the tissue with papain, showed that all of the regions contained only CS (Fig. 4A). When samples of the DEAE-Sephacel fractions that were rich in the polydisperse 80-100 kDa component (possibly decorin) were subjected to β-elimination and then analyzed in agarose-propylene diamine gels, only CS was found in all cases (Fig. 4B). Treatment of these β-eliminated fractions with chondroitinases ABC/AC resulted in complete digestion of the GAGs, indicating that the polydisperse 80-100 kDa component contained CS and not DS.

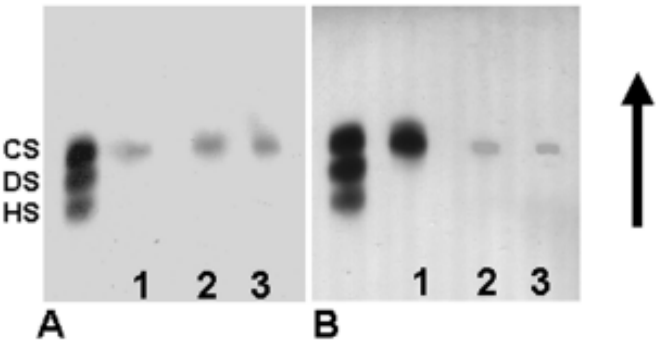

FIGURE 4. Agarose gel electrophoresis of GAGs: A) GAGs obtained after treatment of the tissue with papain. B) GAGs obtained after β-elimination of fractions rich in the 80-100 kDa component. In both cases, a band can be seen at the CS position. On the left are the GAG standards (CS - chondroitin-sulfate, DS - dermatan-sulfate, HS - heparan-sulfate). 1, 2 and 3 correspond to the DC, PL and PM regions, respectively. The arrow indicates the direction of migration.

Discussion

The superficial zone of articular cartilage analyzed here is the most sensitive part of this tissue, since it is the region most exposed to cycles of compression (Oloyede and Broom, 1996). Collagen and PGs are the structural components of cartilage that withstand the tension and compression applied to articular cartilage (Vanwanseele *et al.,* 2002). The organization and distribution of the fibers was seen better when using polarized light microscopy because of the birefringence of collagen bundles. That is produced by the compact, ordered arrangement of the fibers.

In the proximal region, the collagen fibers were arranged predominantly in an antero-posterior direction that corresponded to the main direction of movement of the tibiotarsal-tarsometatarsal joint in ostrich. Although the fibers in the distal region were arranged in several directions, they were less compact than those of the proximal region. These differences probably reflected the higher load supported by the distal region. The biomechanical properties of the distal region require the presence of a mesh of collagen fibers, arranged in several directions, to maintain the high concentration of PGs in this cartilage. Staining with toluidine blue, showed that PGs were more abundant and more homogeneously distributed in the DC region, than in the PM and PL which experience less compression.

The staining of collagen bundles by toluidine blue indicated that the glycosaminoglycans chains of the PGs were arranged parallel to these bundles (Mello and de Campos Vidal, 2003). The distribution of PGs in ostrich articular cartilage was not homogeneous probably because of the non-uniform distribution of the body weight on the cartilage.

The higher content of proteins and sulfated GAGs in the distal region compared to the proximal region confirmed the hypothesis that regions subjected to greater compression have higher amounts of proteins (Ratcliffe and Mow, 1996) and PGs (Kiani *et al*., 2002).

Although there were organizational differences between the articular cartilage of the proximal and distal regions, there were no qualitative differences in the biochemical composition of these regions, since the electrophoretical analysis showed the same banding pattern of proteins. The higher amount of sulfated GAGs found in distal cartilage probably reflected the presence of large PGs, the synthesis of which is stimulated in tissues that support high compressive forces (Kiani *et al.,* 2002).

The polydisperse 67 kDa component may correspond to the small PG fibromodulin, although this PG is reported to have a molecular mass of 59 kDa (Hedbom and Heinegård, 1989). The 67 kDa PG of ostrich may have longer keratan-sulfate (KS) chains than the fibromodulin found in other cartilages.

The 80-100 kDa component had an electrophoretic behavior very similar to the small PG decorin found in bovine cartilage (Heinegård and Pimentel, 1992) and in other tissues such as tendon (Scott and Hughes, 1986) and skin (Kuc and Scott, 1997). Although decorin from soft tissues contains DS, our results indicated that decorin from the different regions of ostrich articular cartilage contained CS, as deduced from digestion with chondroitinase and electrophoresis in agarose gels. The presence of CS in cartilage is expected because aggrecan, a typical CS-PG (Knudson and Knudson, 2001), is abundant in this tissue. However, in our case, the CS was derived from the β-elimination of a polydisperse 80-100 kDa component, similar to the small PG decorin in bovine articular cartilage (Heinegård and Pimentel, 1992).

Another polydisperse component, with a Mr of 250-300 kDa, was equally distributed in proximal and distal cartilages. This component was very similar to the biglycan detected in bovine articular cartilage (Heinegård and Oldberg, 1989). Although the occurrence of this small PG is expected to be greater in regions under high compressive forces, we could not confirm this relationship here, since the intensity of the polydisperse bands with 67, 80-100 and 250-300 kDa were identical for the three regions respectively.

The results reported here should contribute to our understanding of some of the degenerative disorders that affect ostrich articular cartilage.

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