

# Effect of Magnesium Chloride and Guanidinium Chloride on the Extraction of Components of Extracellular Matrix from Chicken Cartilage

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*In order to evaluate the effect of chaotropic agents on proteoglycan and non-collagenous proteins, chicken xiphoid cartilage was treated with guanidine-HCl and MgCl<sub>2</sub> in different concentrations (1M to 5M), and different periods of time (12, 24, 48 and 72hr). The maximum yield of uronic acid was obtained with 3M MgCl<sub>2</sub> (73.3%). Concentrations of 4M and 5M of MgCl<sub>2</sub> showed that much less uronic acid was removed, 55.3% and 38.1% respectively. Extraction with 3M MgCl<sub>2</sub> and 3M guanidine-HCl resulted better efficiency when performed for 48 hr. Analysis by SDS-PAGE of the extracts obtained with guanidine-HCl and MgCl<sub>2</sub> in different concentrations pointed out that most components are equally removed with the two solvents, showing that the extraction with MgCl<sub>2</sub> is an alternative assay to remove non-collagenous proteins from extracellular matrix.*

Key words: proteoglycans – non-collagenous proteins – cartilage – extracellular matrix

Extracellular matrix (ECM) of cartilage is composed of collagen, proteoglycans (PG) and non-collagenous proteins (for review see Heinegård & Paulsson 1984, 1987, Heinegård & Sommarin 1987, Heinegård & Oldberg 1989). The organization and distribution of these components is responsible for the physiological and structural characteristics of each tissue (Vidal 1963, 1964). The study of ECM has been intensively explored in tissues like nasal cartilage (Roughley & Mason 1976, Heinegård et al. 1981, Perin et al. 1987), bovine articular cartilage (Bjele et al. 1974, Rosenberg et al. 1985, 1988), tracheal cartilage from porcine (Antonopoulos et al. 1974), hammerhead fins (Michelacci & Horton 1989) but there are very few works on avian cartilage.

The procedures to extract components of ECM specially PG has laid hold of salts like guanidine-HCl (Gu-HCl) (Hascall & Kimura 1982) and MgCl<sub>2</sub> (Sajdera & Hascall 1969). In avian cartilages 4M Gu-HCl has been used (Oike et al. 1980, McKeown-Longo et al. 1981) as well as NaCl-EDTA (Schulman & Meyer 1970). This latter exhibits a very low dissociative effect.

Considering that the effect of different salts on the extraction of ECM components, depends on the structural arrangement of these components as a consequence of kinds of macromolecules and of the interaction among them that is not the same for all biological material, we in-

vestigated the effect of MgCl<sub>2</sub> and Gu-HCl, under different conditions, on the extraction of PG and non-collagenous proteins.

## MATERIALS AND METHODS

**Extraction** – Fresh xiphoid cartilage was obtained from two months old chicken. The cartilage was dissected free of surrounding tissue and perichondrium, then cut into small slices. Extraction of cartilage samples (1g) was performed by two ways: (a) with 15 volumes of Gu-HCl, 50mM sodium acetate buffer pH 5.8, containing 10mM EDTA; 1mM PMSF; 1mM Benzamidine and (b) 15 volumes of MgCl<sub>2</sub> unbuffered. Both extractions were performed in concentrations ranging 1M to 5M at 4°C for 48 hr with constant shaking. In other case it was used 3M Gu-HCl or 3M MgCl<sub>2</sub> for 12, 24, 48, 72 hr at 4°C. The extracts were separated from the cartilage residues by filtering in clothing. Proteoglycan precipitation was carried out by adding 2,3 volumes of ethanolic Potassium Thiocyanate saturated (KSCN) to the extracts with constant stirring at 4°C. The suspension was left standing for at least 1 hr at 4°C, then centrifuged at 5,000xg for 15 min. The precipitate was washed once with ethanolic KSCN: water (7:3 v/v) and finally three times with 95% ethanol. The washed precipitate was suspended in water in the case of MgCl<sub>2</sub> extraction, and in 50mM sodium acetate buffer pH 7.0 in the case of Gu-HCl extraction.

**Analytical procedures** – Proteins were determined by the Lowry method modified by Hartree

(1972); uronic acid (UA) analyses was carried out after Dische (1947).

**Proteolysis of proteoglycans** – To extract remainder glycosaminoglycans (GAG) in tissue, cartilage residues were treated with papain (40mg/g dry tissue) in 0,03M sodium citrate buffer pH 3.5 containing 0,04M EDTA and 0,08M 2-mercaptoethanol (2-Me). The sample was incubated at 50°C for 24 hr. The digest was clarified by centrifugation and two volumes of ethanol were added to the supernatant. After 24 hr at 4°C, the precipitate was collected by centrifugation, washed with 80% ethanol and with acetone, and dried.

**Uronic acid yield** – To evaluate the yield of UA extracted in each extraction, cartilage residues, after extraction with Gu-HCl or MgCl<sub>2</sub>, were treated with papain. The amount of UA delivered was related to that obtained during Gu-HCl/MgCl<sub>2</sub> extraction (Michelacci & Horton 1989).

**Electrophoresis** – SDS-PAGE (10% acrylamide) was performed according to Zingales (1984). Samples (2.5 µg protein) were prepared in reduced buffer (62mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1mM EDTA, 0.2% 2-Me, 0.01% bromophenol blue) and boiled during three min. Electrode buffer was Tris-glycin (290mM glycin, 25mM Tris and 0.1% SDS). Electrophoresis was done at 25mA for 3 hr. Molecular weight standards used were β-galactosidase (116,000), phosphorylase (97,400), bovine albumin (66,000), egg albumin (45,000) and carbonic anhydrase (29,000). After the running, the gels were transferred to a solution with a fixative, and then were stained by silver method (Blum et al. 1987).

Agarose polyacrylamide gel electrophoresis was carried out with 1.0% agarose and 1.2% acrylamide (Heinegård et al. 1985). The samples (10 µg UA) were prepared in 40mM Tris-acetate pH 6.8; 1mM Na<sub>2</sub>SO<sub>3</sub>; 0.25% SDS and incubated for 2 hr at 37 °C. Afterwards it was added equal volume of 0.05% bromophenol blue, 80% sucrose. Electrode buffer was 10mM Tris-acetate pH 6.8 containing 0.25mM Na<sub>2</sub>SO<sub>3</sub>, 0.06% SDS; 1mM EDTA. Electrophoresis was done at 20mA during 1hr30min. Chondroitin sulfate (CS) and heparan sulfate (HS) were used as standards. The GAGs were stained with 0.1% toluidine blue in 1% acetic acid.

## RESULTS

**Yield of uronic acid after extraction with different concentrations of Gu-HCl and MgCl<sub>2</sub>** – The extractions carried out with different concentrations of MgCl<sub>2</sub>, ranging from 1M up to 5M,

showed that 3M MgCl<sub>2</sub> extracts more UA, with a yield of 73.3% (Table I). This yield was reduced to 55.3% and 38.1% when 4M and 5M were used. Analyses of UA retained in the tissue, showed that only 26.7% of all UA remains in the tissue after treatment with 3M MgCl<sub>2</sub>. In the case of extraction with Gu-HCl also in the same range of concentrations used with MgCl<sub>2</sub> extraction, there was an increase of UA when concentrations of 1, 2 and 3M Gu-HCl were used (Table I). With 4M Gu-HCl there was a decrease of UA and with 5M Gu-HCl the yield of UA was the highest, with 71.3%.

TABLE I

Uronic acid yield in material extracted with MgCl<sub>2</sub> and Gu-HCl in different concentrations for 48 hr. The concentrations values are average ± S.D. (standard deviation) for three samples (n = 3), in each treatment. Yield measurements were carried out taking into account the uronic acid contents in extracted and remainder material

Treatment	Uronic acid			
	Extract (mg/ml)	n	Extract (% of total)	Residue (% of total)
1M MgCl <sub>2</sub>	6.20 ± 0.60	3	35.3	64.7
2M MgCl <sub>2</sub>	4.20 ± 0.75	3	37.3	62.7
3M MgCl <sub>2</sub>	11.00 ± 0.91	3	73.3	26.7
4M MgCl <sub>2</sub>	8.30 ± 0.47	3	55.3	44.7
5M MgCl <sub>2</sub>	5.70 ± 0.25	3	38.1	61.9
1M Gu-HCl	2.77 ± 0.62	3	16.0	84.0
2M Gu-HCl	5.05 ± 0.88	3	37.3	62.7
3M Gu-HCl	7.65 ± 1.05	3	55.3	44.7
4M Gu-HCl	6.55 ± 0.28	3	44.7	55.3
5M Gu-HCl	10.70 ± 0.26	3	71.3	28.7

**Yield of uronic acid after different times of extraction with 3M Gu-HCl and MgCl<sub>2</sub>** – The times of 12, 24, 48 and 72 hr were used to analyze the effect of the time of extraction on the yield of UA. For MgCl<sub>2</sub> extraction the yield of UA started with 56.6% in the time of 12 hr, came up to 57.3% and 66.7%, for 24 hr and 48 hr respectively, but decreased to 43.4% for 72 hr (Table II). In the case of Gu-HCl it was not detected much difference in the different times of extraction. After 12 hr of extraction 56.6% of UA was extracted, followed by 57.3% for 24 hr and 59.3% for 48 hr. For 72 hr 58% of all UA was extracted (Table II).

**SDS-PAGE analysis of extracts obtained with Gu-HCl and MgCl<sub>2</sub>** – The electrophoretic profiles of extracts in the case Gu-HCl (Fig. 1A) and MgCl<sub>2</sub> (Fig. 1B) were similar, but it was observed that in the case of MgCl<sub>2</sub>, there were more bands, although quite discreet, between 58

TABLE II

Uronic acid concentration and yield in material extracted with 3M MgCl<sub>2</sub> and 3M Gu-HCl in different times. The concentrations values are average ± S.D. (standard deviation) for three samples (n = 3), in each treatment. Yield measurements were carried out taking into account the uronic acid contents in extracted and remainder material

Treatment	Uronic acid			
	Extract (mg/ml)	n	Extract (% of total)	Residue (% of total)
12hr MgCl <sub>2</sub>	8.5 ± 0.36	3	56.6	43.4
24hr MgCl <sub>2</sub>	8.0 ± 0.50	3	57.3	42.7
48hr MgCl <sub>2</sub>	8.9 ± 0.25	3	66.7	33.3
72hr MgCl <sub>2</sub>	6.5 ± 0.50	3	43.4	56.6
12hr Gu-HCl	8.5 ± 0.39	3	56.6	43.4
24h Gu-HCl	8.0 ± 0.48	3	57.3	42.7
48hr Gu-HCl	8.9 ± 0.51	3	59.3	40.7
72hr Gu-HCl	8.7 ± 0.50	3	58.0	42.0

kDa and 116 kDa. Some proteins, like 54 kDa and 125 kDa, were equally extracted with MgCl<sub>2</sub> and Gu-HCl, in concentration, of 3, 4 and 5M and much less extracted with 1 and 2M MgCl<sub>2</sub>. Already a 58 kDa protein was not extracted in these two last concentrations, independent of being Gu-HCl or MgCl<sub>2</sub>.

*Agarose polyacrylamide of Gu-HCl and MgCl<sub>2</sub> extracts* – The polydispersity of PG ex-

tracted with Gu-HCl and MgCl<sub>2</sub> may be noticed in agarose polyacrylamide gel. No difference was observed in the different extracts obtained with Gu-HCl (Fig. 2A) and MgCl<sub>2</sub> (Fig. 2B) even when extraction in different times was carried out.

DISCUSSION

To extracted PG and non-collagenous proteins, different methods has been used as 5% NaCl-0.05M EDTA (Schulman & Meyer 1970), 4M Gu-HCl preceded by 0.15M NaCl (Damle et al. 1979), 3M MgCl<sub>2</sub> (Michelacci & Horton 1989) and 4M Gu-HCl (Hascall & Sajdera 1969). The method largely used to extract PG of cartilage and other tissues has been 4M Gu-HCl for 20 hr or 24 hr (Bjelle et al. 1974). In the case of chicken xiphoid cartilage, when Gu-HCl was used, the concentration of 3M was more efficient than 4M Gu-HCl to extract UA, suggesting that 3M Gu-HCl should be the way to extract compounds of ECM from xiphoid cartilage. By the same way, when MgCl<sub>2</sub> was used, the maximum yield of UA was obtained with 3M MgCl<sub>2</sub> and not with 4M and 5M MgCl<sub>2</sub>. This lesser efficiency of UA extraction in concentrations above 3M, for Gu-HCl and MgCl<sub>2</sub>, may result of changes in the solubility and structural stability of the macromolecules due to perturbation at macromolecules-water interface (Damodaran 1989). Another aspect that must be considered

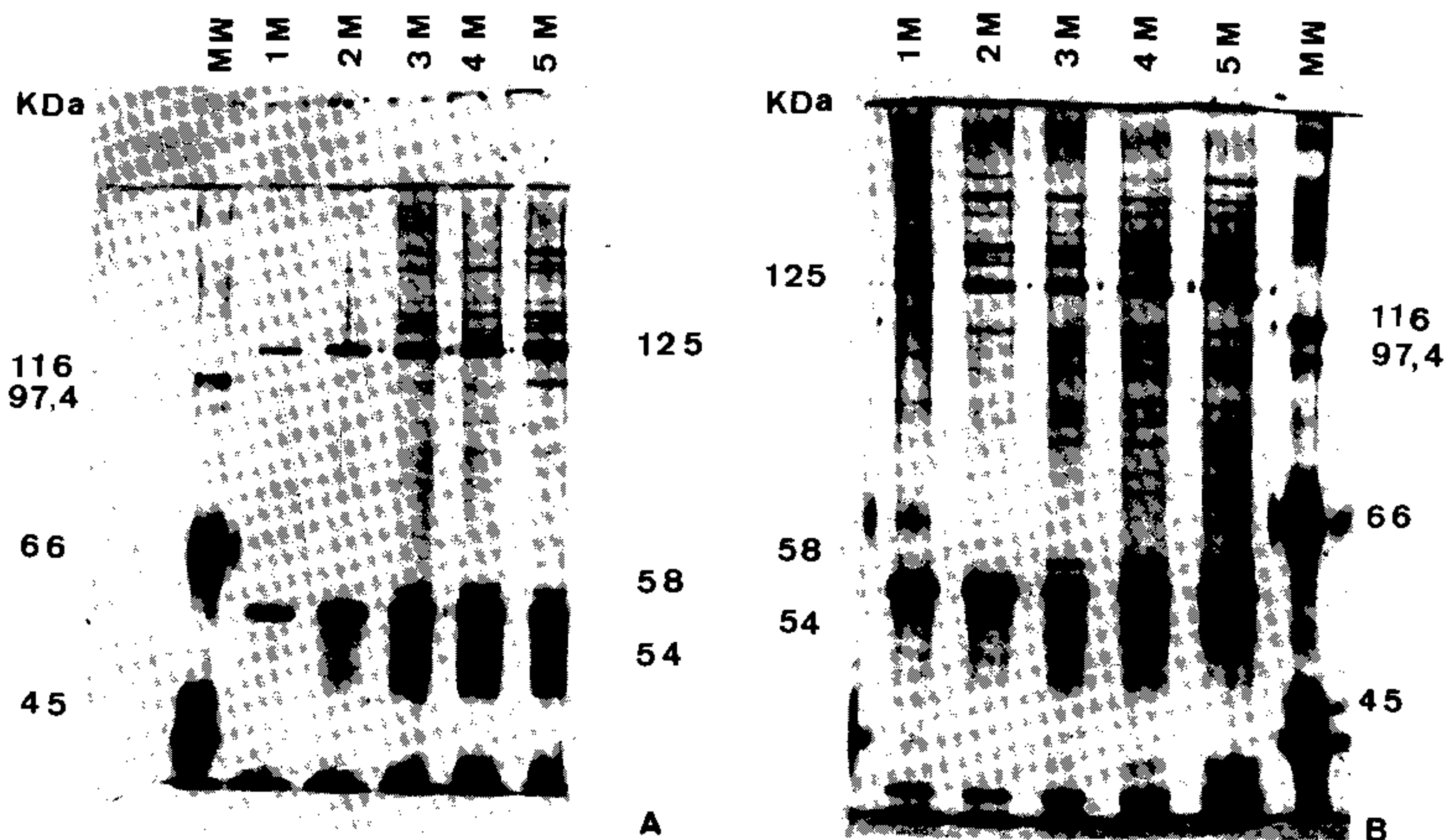


Fig. 1: SDS-PAGE of extract from Gu-HCl (A) and MgCl<sub>2</sub> (B) in concentrations of 1M, 2M, 3M, 4M, and 5M. MW – Molecular Weight Markers.

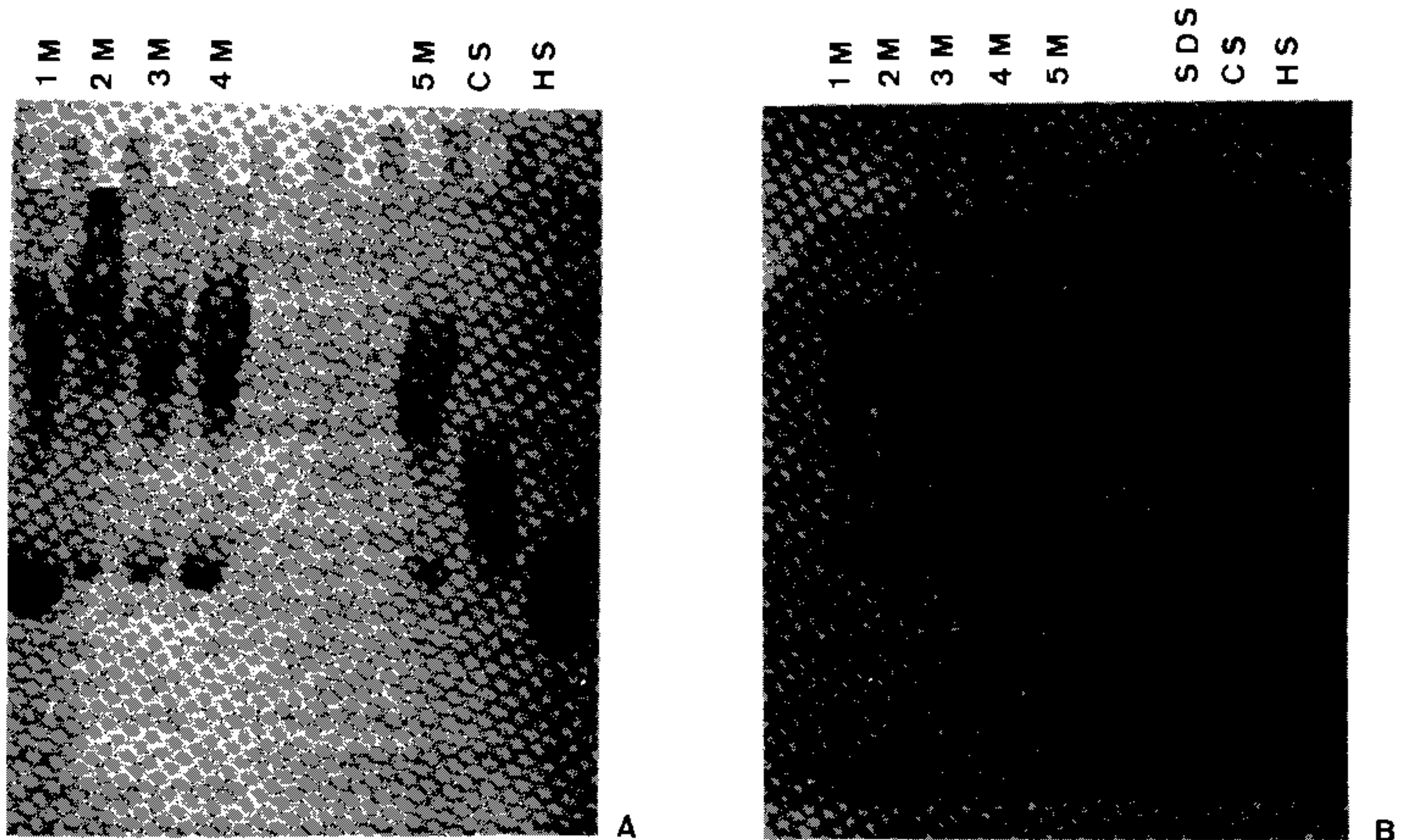


Fig. 2: agarose-polyacrylamide electrophoresis of total extract obtained with GuCl-HCl and MgCl<sub>2</sub> in concentrations of 1M, 2M, 3M, 4M and 5M. CS - Chondroitin sulfate standard. HS - Heparan sulfate standard. SDS - Sodium dodecyl sulfate.

is the nature of the tissue, which results of the distribution and organization of the molecules present in the ECM. This arrangement may influence on the efficiency of the extraction method.

About the time of extraction, when MgCl<sub>2</sub> was used, we had less extracted UA for the time of 72 hr in relation to the time of 48 hr of extraction with the same salt. This result may be due the conformational flexibility of some GAG which in times of extraction superior to 48 hr would allow the interaction between GAG and other proteins (Casu et al. 1988). Although this flexibility is more pronounced in GAG rich in iduronic acids, it also occurs in chains rich in other uronic acids, which facilitates interactions of GAG with non-collagenous proteins, glycoproteins and even with protein core of other PG.

The SDS-PAGE analysis of non-collagenous protein extracted with Gu-HCl and MgCl<sub>2</sub> in different concentrations, showed that most of the components were removed by the two solvents, showing that MgCl<sub>2</sub> may be an alternative procedure to the use of Gu-HCl. The presence of a protein with 42 kDa, which is extracted in larger amount with 3M MgCl<sub>2</sub>, in relation to 3M Gu-HCl, indicates that this protein interacts not so intensely with other components of the ECM.

Samples analyzed in agarose-polyacrylamide showed the presence of just one population of large PG. These PG after papain digestion and analyzed in agarose-polyacrylamide (result not shown), indicated that CS is the most abundant GAG. It is worthwhile to consider that the population and subpopulation of PG may vary with the age (Inerot & Heinegård 1983). In young cartilages predominates CS, in other cartilages has been found PG rich in keratan sulfate (Heinegård & Paulsson 1984). The result obtained here with chicken with 45 days old is according to those authors.

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