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Purification and characterization of hyaluronic acid from chicken combs

Purificação e caracterização do ácido hialurônico obtido da crista de frango

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ABSTRACT

Hyaluronic acid (HA) is an important macromolecule in medical and pharmaceutical fields. The umbilical cord and the chicken comb are some of the tissues richest in this polysaccharide. The profit from obtaining HA from the combs of slaughtered animals is particularly attractive. This work aimed to extract, purify, and characterize HA. The glycosaminoglycan concentration in the chicken comb was found to be about 15 μg of hexuronic acid mg^{-1} of dry tissue. Fractionation using ion exchange chromatography and subsequent identification of the fractions by agarose gel electrophoresis showed that HA corresponded to 90% of the total amount of extracted glycosaminoglycans.

Key words: *glycosaminoglycans; chicken combs, hexuronic acid.*

RESUMO

O ácido hialurônico (AH) é uma importante macromolécula nas áreas médica e farmacêutica. O cordão umbilical e a crista de frango constituem uns dos tecidos mais ricos nesse polissacarídeo. O aproveitamento das cristas dos animais abatidos para a obtenção de HA é particularmente atraente. O presente trabalho teve como objetivo a extração, purificação e caracterização do AH. A concentração de glicosaminoglicanos encontrada na crista de frango foi ao redor de 15μg de ácido hexurônico mg^{-1} de peso seco. O fracionamento por cromatografia de troca iônica e a subsequente identificação das frações por eletroforese de gel de agarose mostrou que o AH corresponde a 90% do total de glicosaminoglicanos extraídos.

Palavras-chave: *glicosaminoglicanos, co-produtos de frangos, ácido hexurônico.*

INTRODUCTION

Poultry production is one of the most important industries in Brazil. In the first trimester of 2011, Brazil produced 1.306 billion chickens (IBGE, 2012). Among the countries in the area, Brazil is the third largest chicken producer in the world market. To maintain success, it is necessary to invest in ways to support low-cost productivity. Moreover, it is necessary to pay special attention to the environment, highlighting the importance of profiting from using the remains from the poultry industry.

The chicken comb is rich in hyaluronic acid (HA) and, being a part of the remains, is discarded with the head to make grease. HA belongs to the glycosaminoglycan (GAG) group, which consists of anionic heteropolysaccharides composed of long, non-ramified and repetitive disaccharide units. HA contains a hexosamine (N-acetyl-D-glucosamine) and uronic acid (D-glucuronic acid). It differs from other GAGs because it does not have N-/O- sulfate groups distributed in its disaccharide units and is not a proteoglycan (HANDEL et al., 2005).

HA is an essential component of the extracellular matrix of vertebrates, and it is also produced by viruses, bacteria and mushrooms. It has several functions, such as joint lubrication and extracellular matrix hydration, and is involved in tumor

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progression, inflammation and regeneration (ALMOND, 2007).

Studies have shown that the hyaluronic acid is not only a lubricant with dermatologic and ophthalmologic applications but also can be used in a control system for drug release, as in anesthesia prolongation in bones and joints (GOLDENHEIN et al., 2001), arthropathy treatment (SUZUKI et al., 2001), chemotherapeutic agents in surgical implants (AEBISCHER et al., 2001), drug release in dental caries (SUHONEN & SCHUG, 2000), controlled antigen release for immunotherapy (PARDOLL et al., 2001), and contact lenses (BEEK et al., 2008), and as a copolymer with anti-thrombotic properties in vascular applications (XU et al., 2008).

The main dermatological application of hyaluronic acid is growing soft tissues through intradermic injections to correct skin problems caused by wrinkles, scars, lip enlargement or other defects (MANNA et al., 1999; INGLEFIELD, 2011).

HA was first isolated from vitreous humor by Meyer and Palmer in 1934. It is a high molecular weight polysaccharide (10^6-10^7Da) with quite a high turnover rate as a component of the cellular matrix. It is catabolized by the enzyme hyaluronidase. Its principal natural sources include the chicken comb, umbilical cord, vitreous humor, and synovial fluid (DEVLIN, 2000; ALMOND, 2007). This work aimed to extract, purify and characterize hyaluronic acid from the chicken comb of 48-day-old male and female chickens.

MATERIALS AND METHODS

Source material

Chicken combs were provided by the Pena Sul slaughterhouse (Caxias do Sul, RS). Forty kilograms of combs were collected from a 50:50 population of 48-day-old male and female chickens. The combs were submerged in hot water and then frozen at -18ºC until the experiments were performed. The combs were analyzed without gender distinction. The trials were conducted at the Laboratory of Connective Tissue at the University Hospital Fraga Filho at the Federal University of Rio de Janeiro.

Extraction of the total glycosaminoglycans from the chicken combs

The combs were crushed and placed in acetone for dehydration and delipidation. Subsequently, they were dried and weighed (100g) for each extraction (nº3). As a first step in the extraction, delipidation was conducted in a chloroform and methanol solution (2:1, v/v) for 24h at 25ºC. The tissues were dried and hydrated in digestion buffer (100mM sodium acetate pH 5.0, 5.0mM cysteine and 5.0mM disodium-EDTA) in a ratio of 2.0mL of buffer to 100mg of dry tissue. After hydration (24h at 4ºC), a solution of papain in digestion buffer (20mg mL^{-1}) was added in the ratio of 0.5mL to 100mg of dry tissue. The mixture was incubated (24h at 60ºC), centrifuged at 3200rpm for 30min, and the supernatant was removed. The pellet was discarded. Then, 10% CPC was added to the supernatant in the ratio of 0.2mL to 100mg of dry tissue and left for 24h at 25ºC. The sample was centrifuged (3200rpm / 30min), the supernatant was discarded, and the pellet washed with 3.0mL of 2.0M NaCl and absolute ethanol (100:15 v/v). Absolute ethanol (2:1, v/v) was added, and the mixture was incubated (24h at -16ºC). Next, centrifugation was performed (3200rpm / 30min), the supernatant was discarded, and the pellet was washed once with 10mL of 80% ethanol. The solution was centrifuged again (3200rpm / 30min), the supernatant was discarded and the pellet was dried (24h at 25ºC). The final solid was re-suspended in 5mL of distilled water, and the total content of the GAGs was measured by the hexuronic acid percentage in the solution using a carbazole reaction (DISCHE, 1946).

Fragmentation of the glycosaminoglycans

The glycosaminoglycans from the chicken comb (~500 g in hexuronic acid) were applied to a Mono-Q column coupled to a FPLC system, equilibrated using 20mM Tris-HCl (pH 8.0) and submitted to a NaCl (0 to 1.5M) linear gradient in the same buffer. The column had a flow of 1mL min^{-1}, and 0.5mL fractions were collected. They were evaluated by the content of hexuronic acid (carbazole reaction) and the metachromasia produced by the glycosaminoglycans sulfated in the presence of 1.9-dimethylmethylene blue (FARNDALE et al., 1986). The salt concentration was measured by the conductivity. The fractions containing glycosaminoglycans, as indicated by the uronic acid dosage, were gathered and precipitated with 3 volumes of absolute ethanol.

Agarose gel electrophoresis

The total glycosaminoglycans samples from the chicken comb and the fractions obtained through the ionic exchange chromatography were applied (~5µg in hexuronic acid) to a 0.5% agarose gel prepared in a 50mM diaminopropane (pH 9.0) buffer and submitted to 110V for almost 1h (DIETRICH & DIETRICH, 1976). The GAGs in the gel were fixed with 0.1% cetavlon (N-cetyl-N,N,N-trimethylammonium bromide in water) and then dyed with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5,v/v/v) to reveal

the sulfated glycosaminoglycans. After identifying the metachromatic fractions, the gel was dyed with 0.005% Stains-All in 50% ethanol (VOLPI et al., 2005; VOLPI & MACCARI, 2006). The standards were human thoracic aorta GAGs and hyaluronic acid (Sigma-Aldrich, USA).

^{13}C-NMR spectroscopy

The ^{13}C-NMR spectra were obtained using a Bruker DPX 400MHz spectrometer. A D_2O solvent was used to acquire the ^{13}C-NMR spectra (reference: δ=0ppm, 4-dimethyl-4-silapentane-1-sulfonate).

The experimental parameters used to acquire the spectra were as follows

Bruker DPX-400: SF 400.13MHz spectrometer for ^{1}H and 100.23MHz for ^{13}C; pulse width 90°: 8.0 µ s (^{1}H) and 13.7 µs (^{13}C); acquirement time 6.5s (^{1}H) and 7.6s (^{13}C); spectral window 965Hz (^{1}H) and 5000Hz (^{13}C); scanning number 8-32 for ^{1}H and 2000-20000 for ^{13}C, depending on the compost; number of points: 65536 with digital resolution Hz/point ^{1}H equal to 0.677065 (^{1}H) and 0.371260 (^{13}C); temperature: 50ºC.

RESULTS AND DISCUSSION

GAG concentration in the chicken combs

The powder obtained from the chicken combs corresponded to ~16% of the net weight. The total glycosaminoglycan concentration was 15µg of hexuronic acid mg^{-1} of dry tissue. This value is much lower than that reported by NAKANO & SIM (1989) and NAKANO et al. (1994), which was 42.1 µg of hexuronic acid mg^{-1} of dry tissue. However, in that study, the glycosaminoglycans were extracted from 52-week-old animals, while in this study, they were obtained from 48-day-old animals. NAKANO et al. (1994) reported that hexuronic acid in the wattle of 52-week-old chickens was 19.1 µg mg^{-1} of dry tissue. This value is closer to the amount we found in the chicken combs.

According to NAKANO et al., (1994), the combs of older males possess greater amounts of hyaluronic acid. In addition, scalding the combs may decrease the HA concentration (SZIRMAI, 1956; BALAZS et al., 1958; SWANN, 1968). Nevertheless, HA extraction may be worthwhile due to the number of chickens slaughtered in slaughterhouses.

In the first trimester of 2011, 1.306 billion chickens were slaughtered in Brazil (data from IBGE 2012). Considering that each chicken comb has an average of 3 grams of humid weight, the amount of combs generated by the poultry industry would be 3918 tons. In this study, we obtained 2.0 µg of hexuronic acid mg^{-1} of humid tissue extracted from chicken combs. Because hyaluronic acid corresponded to 90% of the extracted hexuronic acid, an estimated 7.05 tons of hyaluronic acid could have been extracted during the first trimester of 2011. It must be highlighted that hyaluronic acid has a high market value (US $65.00 100mL^{-1}) and is not commercially produced in Brazil (OGRODOWSKI, 2006). The chicken comb, which is part of the remains of the poultry industry, is potentially a great source of hyaluronic acid for use in the medical, pharmaceutical and cosmetic industries.

Fragmentation of the GAGs extracted by ion exchange chromatography

The total GAG extract from the chicken combs was fractionated using a Mono-Q column. The results are shown in figure 1. We observed a large peak corresponding to ~90% of the total hexuronic acid in the sample that eluted with ~400mM NaCl without showing any significant metachromasia. These properties are characteristic of HA when it is applied to this column. We also observed a fraction with metachromatic properties when eluted with a NaCl concentration greater than 1M, which indicates the presence of sulfated GAGs in the analyzed sample.

The non-sulfated glycosaminoglycans did not change color in the presence of DMB due to the deprotonation of the carboxyl groups. This finding shows that other specific factors beyond polymer charge density, such as sulfate groups, are required for metachromasia in the presence of DMB. In solutions with balance between monomer and dimer colorants, the position of balance does not affect the presence of sulfated glycosaminoglycans, but the interaction with monomer and dimer colorants with polyanions produces a new species of absorption and removes the solution color. HA interacts with the dimer colorant to build a new species of absorption or extinguish the monomers and dimers, creating a new balance with metachromasia (TEMPLETON, 1988).

Qualitative analysis of the extracted GAGs

To identify the GAG species present in the fractions recovered using ion exchange chromatography, the samples and the total extract were gathered and analyzed using agarose gel electrophoresis. Figure 2a shows the gel dyed with toluidine blue, which is used to identify the presence of sulfated species. For the total extract, we observed the presence of two bands with metachromatic coloring and electrophoretic mobility that were similar to dermatan sulfate and chondroitin sulfate. We also observed a non-metachromatic fraction migrating between dermatan sulfate and the heparan sulfate

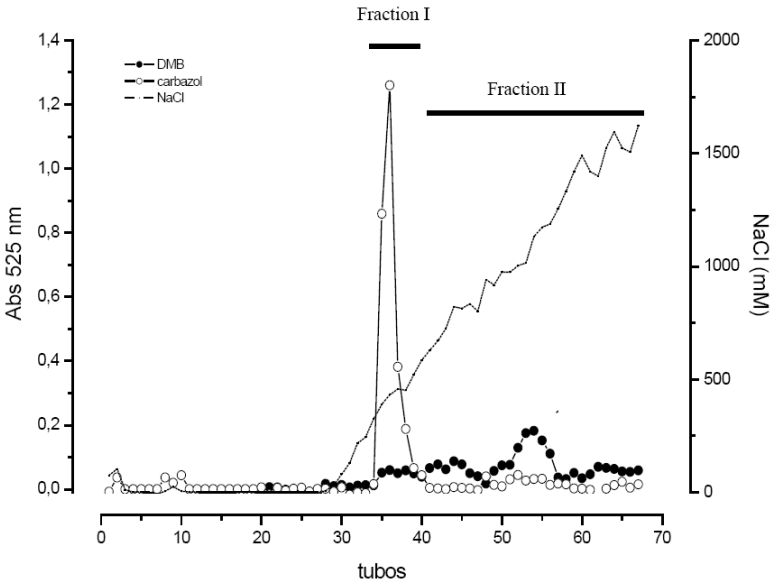

Figure 1 - Fractionation of the GAGs extracted from the chicken combs using ion exchange chromatography. The fractions were analyzed through metachromasia (●) and hexuronic acid content (○). The horizontal bars show both fractions.

standard. The latter had a strong blue color when using Stains All, (Figure 2b), indicating that it is hyaluronic acid. When analyzing the fractions obtained through ion exchange chromatography, we eluted a greater quantity of hyaluronic acid using 0.4M NaCl. The remaining 10% was mainly composed of dermatan sulfate and chondroitin sulfate chains. Similar results were found by LAGO et al. (2005) when isolating large amounts of hyaluronic acid from human umbilical cords; thus, both umbilical cords and chicken combs are the main sources of HA.

^{13}C-NMR spectroscopy

The total amount of GAG extracted from the chicken combs was identified using ^{13}C NMR spectroscopy. The ^{13}C-NMR spectra were acquired at 100.23MHz in a Bruker DPX400 spectrometer at 50ºC. The sample was prepared by dissolving 5mg of the solid in 0.5mL of D_2O (pH=6.0). The chemical shifts were measured in relation to the internal standard 4,4-dimethyl-4-silapentane-1-sulfonate. In the ^{13}C-NMR spectra of this sample, we found signals similar to those previously reported by BOCIEK et al. (1980) and VOLPI

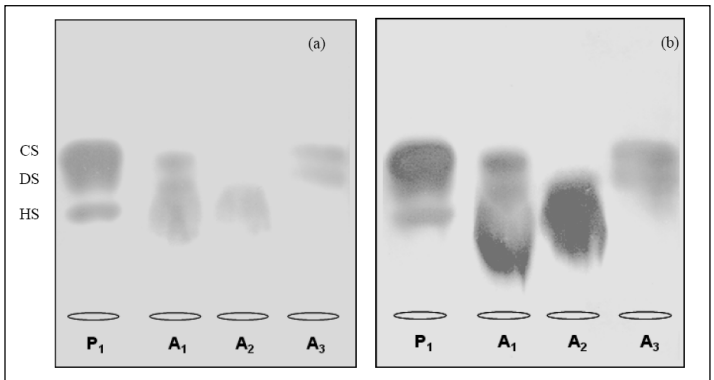

Figure 2 - Agarose gel electrophoresis of total GAG and fractions recovered through ion exchange chromatography. (a) Stained with toluidine blue; (b) Stained with toluidine blue and Stains-All. P – standard, A_1 – total GAG, A_2 – fraction I, A_3 – fraction II, CS – chondroitin sulfate, DS – dermatan sulfate.

& MACCARI (2003) for HA from other sources under similar conditions, including pH. LAGO et al. (2005), working with human umbilical cord, using ^{13}C NMR spectroscopy identified carboxyl and acetamide groups at 173.4 and 144.5ppm, respectively, two anomeric carbons at 100.1 and 102.7ppm and an acetamide carbon at 22.1ppm. The signals at 53.9 and 60.4ppm are from the C2 and C6 carbons of the glucosamine residue. All of the resonances described support the presence and purity of HA from the umbilical cord, confirming the results found in this experiment under similar conditions.

In the ^{13}C-NMR spectra (Figure 3), the chemical shifts were determined for the carbons from the β-D-glucuronic acid units, and a signal was observed at 176.96ppm for COO^-. The signals were verified at 106.06ppm for C-1, at 83.0ppm for C-4, and at 79.41ppm for C-5. Signals corresponding to C-3 at 76.70ppm and C-2 at 75.59ppm were also observed. For the 2-acetamide-deoxy-β-D-glucopyranoside units, a signal was observed at 177.87ppm for the C-2 carbons, and we identified signals at 103.44, 85.68, 78.45, 71.62, 63.73 and 57.33ppm corresponding to the C-1, C-3, C-5, C-4, C-6 and C-2 carbons, respectively. At 25.56ppm, a characteristic shift for the methylic carbon was observed. The other signals found in the spectra were classified as matrix impurities.

CONCLUSION

The results obtained shows that the applied methodology is effective for extracting and purifying hyaluronic acid. The quantitative and qualitative analysis of the GAGs showed that the chicken comb is predominately composed of HA (90%), with the presence of sulfated GAGs in a lower concentration (10%). Thus, the hyaluronic acid obtained from chicken combs may be used as a co-product from the poultry industry for research and clinical applications.

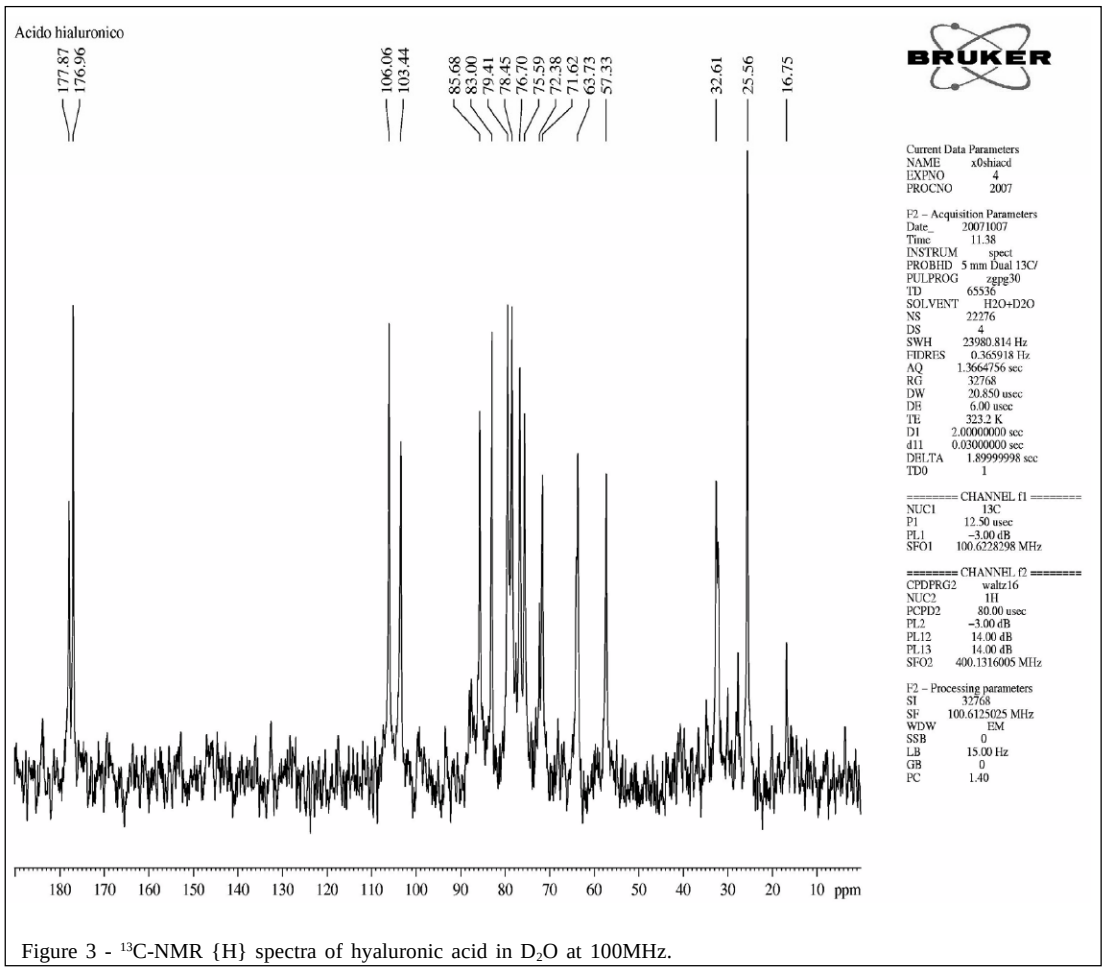

Figure 3 - ^{13}C-NMR {H} spectra of hyaluronic acid in D_2O at 100MHz.

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