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ORIGINAL ARTICLE

Glycosaminoglycans from chicken muscular stomach or gizzard

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Abstract Glycosaminoglycans (GAGs) were prepared from the muscular stomach or gizzard of the chicken. The content of GAGs on a dry weight basis contains 0.4 wt.% a typical value observed for a muscle tissue. The major GAG components were chondroitin-6-sulfate and chondroitin-4-sulfate (~64 %) of molecular weight 21–22 kDa. Hyaluronan (~24 %) had a molecular weight 120 kDa. Smaller amounts (12 %) of heparan sulfate was also present which was made of more highly sulfated chains of molecular weight of 21-22 kDa and a less sulfated low molecular weight (< 10 kDa) heterogeneous partially degraded heparan sulfate. Chicken gizzard represents an inexpensive and readily available source of muscle tissue-derived GAGs.

Keywords Glycosaminoglycans · Compositional analysis · Heparan sulfate · Chondroitin sulfate · Hyaluranan · Chicken gizzard

Abbreviations

GAG	Glycosaminoglycan
HS	Heparan sulfate
CS	Chondroitin sulfate
KS	Keratan sulfate
MWCO	Molecular weight cut off
HPLC-MS	High performance liquid chromatography and mass spectrometry
DMMB	1,9-dimethylmethylene blue
AMAC	2-aminoacridone
GPC	Gel permeation chromatography

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Introduction

Glycosaminoglycans (GAGs) are linear polyanionic polysaccharides that are found ubiquitously in all animals [1–3]. The glycosaminoglycan family includes chondroitin sulfates, heparan sulfates, hyaluronan and keratan sulfate. Each class of glycosaminoglycan are comprised of a characteristic, variably *O*-sulfo group substituted, disaccharide-repeating unit. For example, chondroitin sulfate (CS) is comprised of repeating →4) β-D-glucuronic acid (or α-L-iduronic acid) (1 → 3) β-D-*N*-acetylgalacatosamine (1 → and heparan sulfate (HS) is comprised of repeating →4) α-D-glucuronic acid (or α-L-iduronic acid) (1 → 4) α-D-*N*-acetylglucosamine(or *N*-sulfoglucosamine) (1→. Hyaluronan (HA) is →4) β-D-glucuronic acid (1 → 3) β-D-*N*-acetylglucosamine (1 → and contains no sulfo groups.

GAGs perform critical functions in developmental biology [4] and in normal physiology and pathophysiology [5] through their ability to bind to and modulate the activity of a wide array of proteins [6–8]. Through these interactions GAGs impact vascular biology [9], cancer [10], infectious diseases [11], inflammation [8, 12], and promote signaling [13].

Our research group has been interested in characterizing GAGs isolated from various tissues and species [14–22].

These not only impact the biology of these animals, they also offer potential new sources of GAGs as pharmaceutical and nutraceuticals and potentially unusual GAG structures. GAGs of avian species used as human food (chickens and turkeys) have also been studied as they represent a widely available tissue source for GAG production [23–25].

The current study focuses on the GAG composition of chicken gizzard. The gizzard is a muscular stomach common to certain vertebrate and invertebrate animal species that typically contains sand or small stones to help in the grinding of food to assist in its digestion. While the GAGs of human stomach have been studied [26], little if anything is known about the role of GAGs in the chicken gizzard but there is an old report of such molecules in the gizzard of earthworms [27]. This information on the composition and structure GAGs derived from chicken gizzard should help broaden our understanding of the biochemistry and function of these biomacromolecules in this tissue.

Materials and methods

Materials

Sodium cyanoborohydride ($NaCNBH_4$), acetic acid, 1,9-dimethylmethylene blue (DMMB), 2-aminoacridone (AMAC), and carbazole were purchased from Sigma Aldrich (St. Louis, MO). Methanol (HPLC grade), ammonium acetate (HPLC grade), and dimethyl sulfoxide (DMSO) were from Fisher Scientific (Springfield, NJ). Actinase E was from Kaken Biochemicals (Japan). Heparin lyase I, II, III (EC # 4.2.2.7, 4.2.2.-, and 4.2.2.8, respectively) and chondroitin lyase ABC (EC #4.2.2.20) were produced in *Escherichia coli as* recombinant enzymes in our laboratory as previously described [28]. Chicken gizzards were obtained at the local supermarket.

Extraction of GAGs from chicken gizzard

After cutting the tissue (~120 g gizzards) into small pieces (<5 mm) with a scalpel, the gizzard tissue was freeze-dried (~50 g dry weight), suspended in 200 mL phosphate buffer (pH 7) and then subjected to proteolysis at 55 °C for 48 h by adding 10 % (volume) of actinase E (20 mg/mL in water). After centrifugation at 6500×*g* for 30 min to remove particulates, the supernatant was recovered and concentrated 20-fold by rotary evaporation. The GAGs were precipitated from the concentrated supernatant by adding three-volumes of cold ethanol. The GAGs were then recovered by centrifugation (8000×*g*, 10 min), dissolved in 50 mL of water dialyzed using a cellulose membrane (molecular weight cut off (MWCO) of 3500) against distilled water for 48 h and freeze-dried.

Separation of GAGs

The crude GAGs were firstly fractionated by anion-exchange chromatography using a HiPreQ Sepharose Fast Flow column (16 × 10 mm) coupled to an AKTA FPLC system (GE Life Sciences, Pittsburgh, USA), eluted with linear (Fig. 1a) and step-wise gradient (Fig. 1b) of NaCl. The fractions were assayed for uronic acid content by the carbazole method [29]. The major GAGs containing fractions were pooled, desalted by dialysis (MWCO 3500) and freeze–dried.

The isolated total GAGs (containing a mixture of HA, CS and HS) were divided to two equal potions in buffer of 100 mM ammonium acetate containing 10 mM $CaCl_2$ (pH 7.5). The first portion was digested with chondroitin lyase ABC (20 mU in 25 mM Tris, 500 mM NaCl, 300 mM imidazole buffer, pH 7.4) able to break down CS and HA. The second portion was digested with a mixture of heparin lyase I, II, III (40 mU/each enzyme in 25 mM Tris, 500 mM NaCl, 300 mM imidazole buffer, pH 7.4) to specifically break down HS. After digestion was complete, the samples were heated at 100 °C for 15 min and centrifuged (8000×*g*, 15 min) to remove the thermally inactivated enzymes, the supernatant was filtered through a 3 kDa molecule MWCO spin column to remove the disaccharide and oligosaccharide products of enzyme digestion, washed twice with 100 μL distilled water, the retentate containing intact GAG was dissolved in water and freeze-dried.

Disaccharide analysis of GAGs in chicken stomach

The intact GAG samples (200 μg) were next completely depolymerized using chondroitin lyase ABC (20 mU) and heparin lyase I, II, III (40 mU/each enzyme) in buffer of 100 mM ammonium acetate containing 10 mM $CaCl_2$ (pH 7.4) and diluted to a final volume of 200 μL. Reacting mixtures were incubated at 35 °C for 12 h. After the reaction finished, the enzymes were removed by centrifugation through a 3 kDa MWCO spin column and the filter unit was washed twice with 100 μL distilled water and the filtrate that contained the disaccharide products was recovered and freeze-dried.

After complete depolymerization to disaccharide products and AMAC labeling, disaccharide analysis of the fractions, which contained CS and HA or HS, was performed using high performance liquid chromatography-mass spectrometry (HPLC-MS). The dried samples were AMAC-labeled by adding 10 μL of 0.1 M AMAC in DMSO/acetic acid (17/3,*V*/V) incubating at room temperature for 10 min, followed by adding 10 μL of 1 M aqueous $NaBH_3CN$ and incubating for 1 h at 45 °C. A mixture containing HS or CS disaccharides standards was also prepared at a concentration of 1250 ng/mL and similarly AMAC-labeled for use as a standard. After the

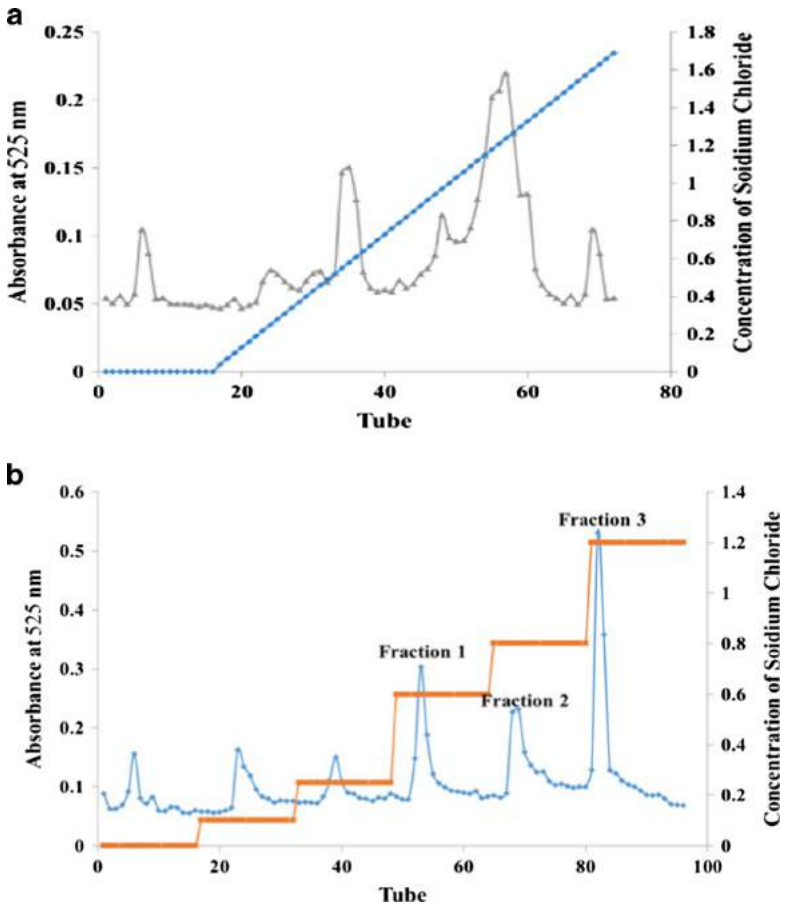


Fig. 1 Purification of chicken gizzard GAGs by anion-exchange chromatography using a HiPre Q Sepharose Fast Flow column. **a** The linear elution curve of chicken gizzard GAGs; **b** The stepwise elution curve of gizzard GAGs

AMAC-labeling reaction, the samples were centrifuged and each supernatant was recovered and an equal volume of DMSO:aceticacid:distilled water (17:3:20) was added to each. Samples were stored in a light resistant container at room temperature until analyzed using HPLC-MS.

LC was performed on an Agilent 1200 LC system at 45 °C using an Agilent Poroshell 120 ECC18 (2.7 μm, 3.0 × 50 mm) column. Eluent A was 50 mM NH_4OAc, and the eluent B was methanol. The mobile phase passed through the column at a flow rate of 300 μL/min. The gradient of eluent B increased from 5 % to 30 % in the first 20 min, and rose to 50 % eluent B in the following 10 min, then to 100 % eluent B in 1 min, and a 9 min flow of 100 % eluent B was applied to elute all compounds. The column effluent entered the source of the ESI-MS for continuous detection by MS. The electrospray interface was set in negative ionization mode with a skimmer potential of −40.0 V, a capillary exit of −40.0 V, and a source of temperature of 325 °C, to obtain the maximum abundance of the ions in a full scan spectrum (150–1500 Da, 10 full scans/s). Nitrogen was used as a drying (5 L/min) and nebulizing gas (20 psi).

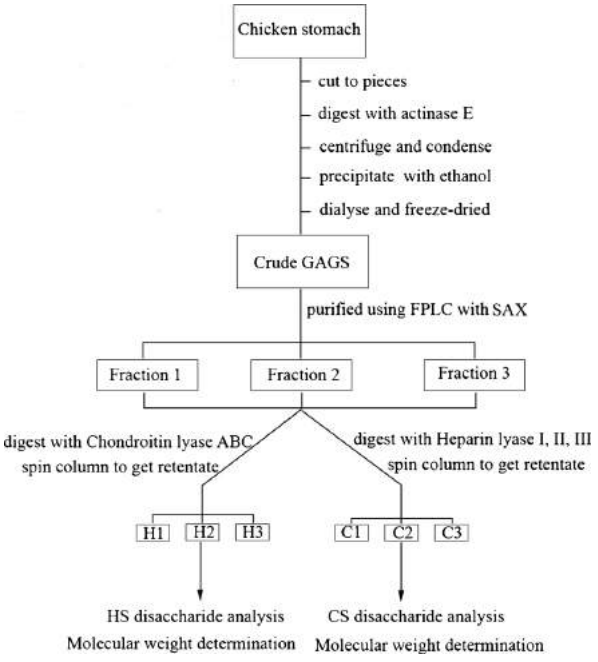


Fig. 2 Diagram of process flow chart

Molecular weight determination of GAGs

The molecular weights of GAGs prepared from chicken gizzard was determined by HPLC-gel permeation chromatography (GPC) using United States Pharmacopeia (USP) heparin standard and HA molecular weight standards. Chromatography used a mobile phase of 0.1 M ammonium acetate and 0.02 % sodium azide in water was filtered through a 0.22-μm membrane. The chromatography columns were a TSK guard column (6 mm × 4 cm), a TSK SWXL 4000 column (7.8 mm × 30 cm) and a TSK SWXL 3000 column (7.8 mm × 30 cm) in series, at 30 °C. The flow rate was 0.6 mL/min. Refractive index detection was used, at the same temperature as the columns. Data were analyzed using SEC specialist (Shimadzu LC solution GPC software, Japan).

Results

Chicken gizzard contains abundant connective tissue and on extraction yielded of the total ~ 0.4 % (GAG wt./dry wt.) total GAGs. The crude GAG extract was first purified using a Q Sepharose Fast Flow column eluted with two column volumes of water followed by 10 column volumes of a linear sodium chloride gradient from 0 to 2 M aq. sodium chloride (Fig.1a). Based on the results of linear elution and the position of the peaks, we optimized the gradient elution conditions. Six peaks contained uronic acid. The first three peaks mostly contained proteins that could be glycosylated with an uronic acid containing glycan. The three major fractions containing GAGs were eluted using a stepwise elution with 0.6, 0.8 and 1.2 M NaCl (Fig. 1b). The three GAG fractions were dialyzed, lyophilized and freeze-dried.

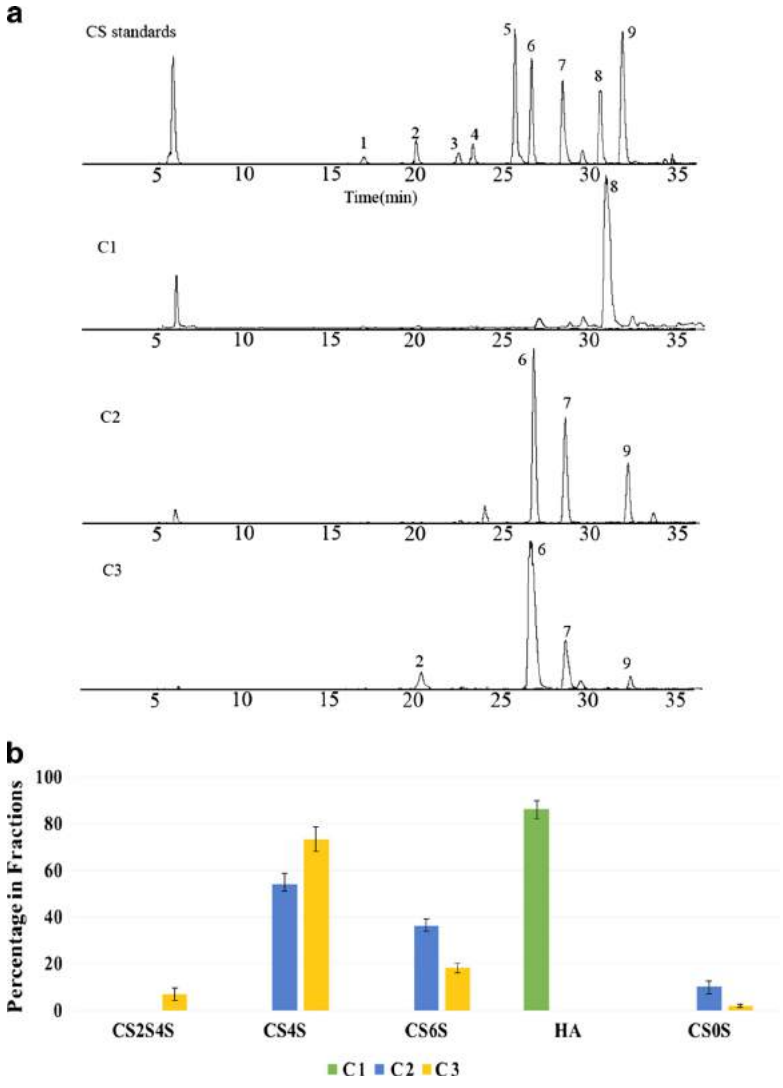


Fig. 3 Disaccharide analysis of chondroitin sulfate and hyaluronan GAGs. **a** Disaccharides analysis of CS fractions (1–9 corresponds to CS_{Tris}, CS_{2S4S}, C_{S2S6S}, CS_{4S6S}, CS_{2S}, CS_{4S}, CS_{6S}, HA and CS_{0S}; **b** Disaccharide composition of CS fractions C1, C2, and C3

It is hard to purify different GAGs using only anion exchange chromatography. The three fractions collected were, therefore, subjected to enzymatic digestion to remove specific GAGs. Chondroitin lyase ABC digest CS and HA but does not act on HS. In contrast, heparin lyases I, II, III digest HS but do not act on CS or HA. In this step, each fraction was divided in half and before enzymatic treatment to isolate either CS and HA or HS. The three fractions afforded six samples three of which were subjected to chondroitin lyase ABC (H1, H2 and H3) and three subjected to heparin lyase I, II, III (C1, C2 and C3) (Fig. 2).

Disaccharide composition of GAGs

Next disaccharide analysis was performed on the six samples obtained (Fig. 3a). Sample C1 was mainly consisted of HA disaccharide. Samples C2 was CS comprised of CS0S, CS4S and CS6S in a ratio of 1.0:5.5:3.8. Sample C3 had a composition with a higher level of sulfation comprised of CS0S, CS4S, CS6S and CS2S4S in a ratio of 1:75:19:5. Not as C2, C3 contained additional 5 %. The composition and content of C1, C2 and C3 was shown in Fig. 3b.

The HS fractions were next analyzed. These samples contained every HS disaccharide except for HS_{2S6S} (Fig. 4a). Most abundant disaccharide was HS_{0S}, which accounted more than a half of the HS GAG. Sample H1 contained nearly 75 % $HS_{0S}$with small amounts of HS_{NS}, HS_{NS2S} and HS_{TriS}, and trace amounts of HS_{6S}, HS_{NS6S} and HS_{2S} (Fig. 4b). As the ionic strength increased from H1, H2 to H3, the content of HS_{0S} decreased, and the contents of HS_{NS}, HS_{NS2S}, HS_{NS6S}, HS_{6S} and HS_{2S} increased. HS_{TriS}, most commonly associated with heparin structure, was only observed was observed in small (~5 %) but equal amounts in all fractions. Fraction H3 contained 8 % of the rare HS_{2S} that was observed in much smaller amounts in fractions H1 and H2.

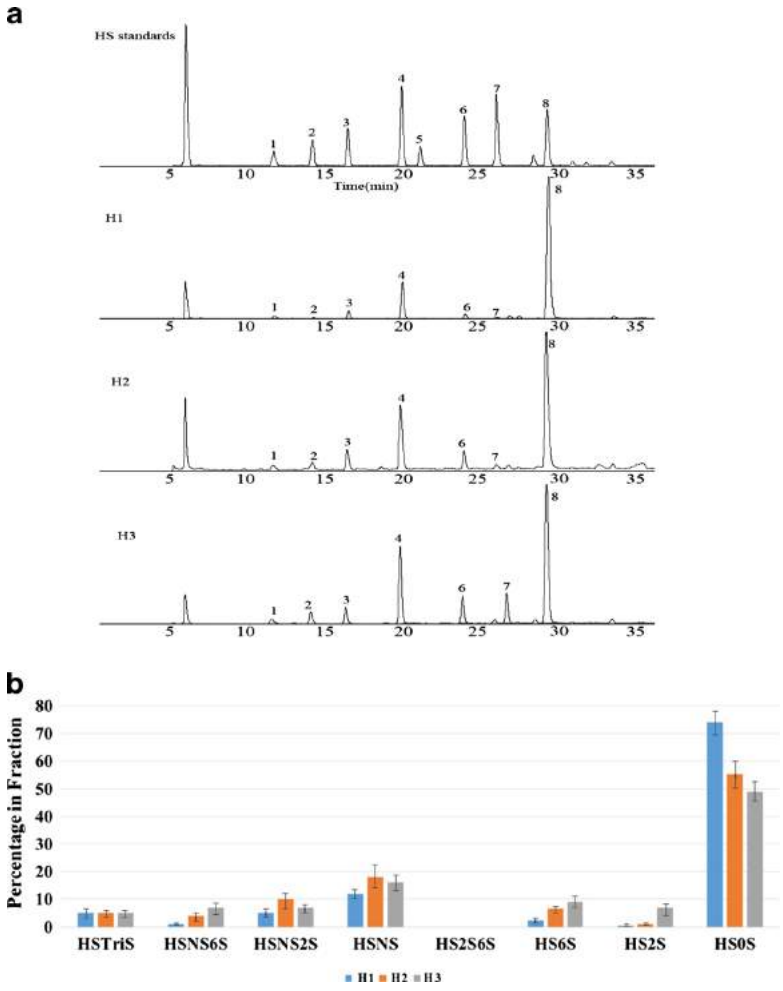
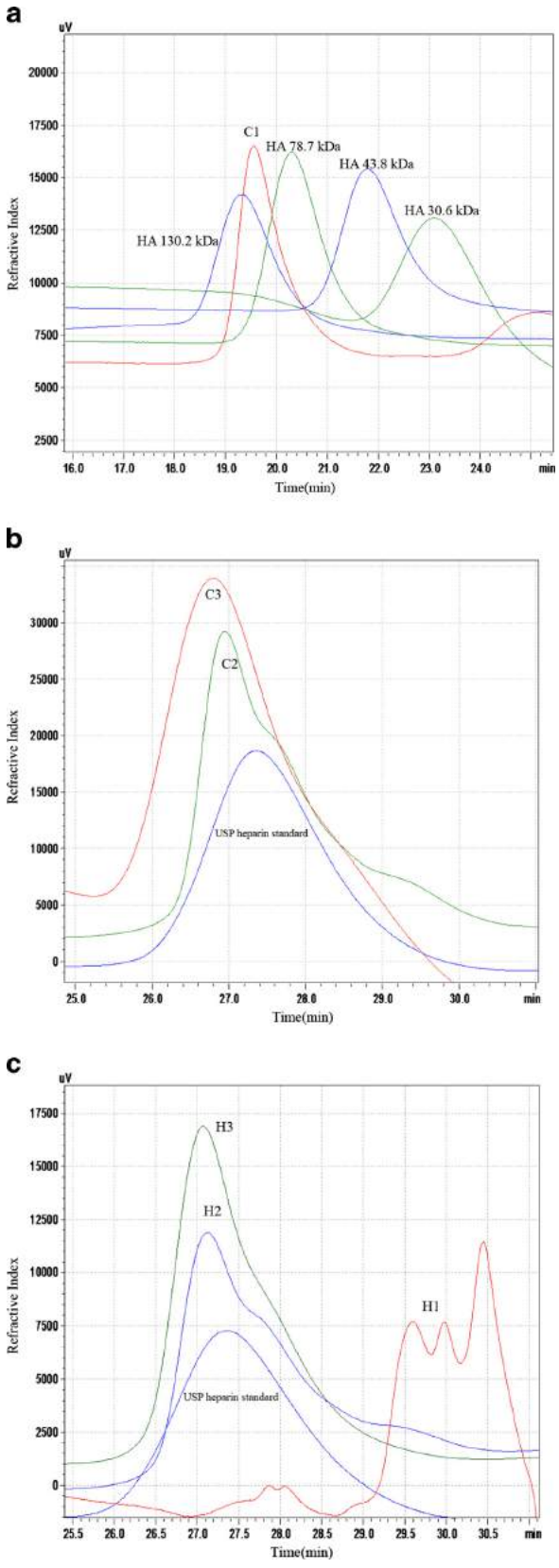


Fig. 4 Disaccharide analysis of heparan sulfate GAGs. **a** Disaccharides analysis of HS fractions (1–8 correspond to HS_{TriS}, HS_{NS6S}, HS_{NS2S}, HS_{NS}, HS_{2S6S}, HS_{6S}, HS_{2S} and HS_{0S}; **b** Disaccharide composition of HS fractions H1, H2 and H3



◀ **Fig. 5** Analysis of the molecular mass of GAGs isolated from chicken gizzard. **a** HPLC-GPC chromatogram of HA; **b** HPLC-GPC chromatogram of CS; **c**HPLC-GPC chromatogram of HS

Molecular weight of GAGs

Fraction C1, composed of HA had the largest molecular mass among all of the GAGs. HA standards having different molecular masses (130.2 kDa, 78.7 kDa, 43.8 kDa and 30.6 kDa) were used to generate a calibration curve to determinate the molecular mass of the HA in fraction C1. From the HPLC-GPC chromatograms (Fig. 5a), fraction C1 consisting of pure HA had a molecular mass of 120.0 kDa.

The molecular mass of fractions C2, C3 and H1–3 relied on the USP calibration heparin as the molecular weight standard. Fractions C2 and C3 had molecular weights of 20.0 kDa and 22.0 kDa, respectively (Fig. 5b). Fractions H2 and H3 had nearly identical molecular masses of 21.0 kDa and 22.0 kDa, respectively, and fraction H1 showed three overlapped peaks in the chromatogram and a molecular mass of ~ 10.0 kDa (Fig. 5c).

Discussion

Dried chicken muscular stomach or gizzard on a dry weight basis contains 0.4 wt.% GAG of this GAG12.2 % was HS corresponding to 0.048 wt.% HS. A previous study demonstrated that whole mouse has 0.012 wt.% HS and mouse heart muscle tissue contains 0.08 wt.% HS on a defatted dry weight basis [15, 16]. The muscular stomach or gizzard of chicken, thus, shows a reasonable GAG content. The major GAG of the muscular stomach or gizzard of chicken was CS corresponding to 64 % of the GAG content (Fig. 6). There were two fractions contained a CS of molecular weight of 21–22 kDa, fairly typical of commercial CS prepared from bovine trachea [30]. The disaccharide composition shows prominent amounts of $CS_{4S}$and $CS_{6S}$with small amounts of CS_{0S} and CS_{2S4S}consistent with CS prepared from mammalian sources. HA accounted for 24 % of the total GAGs and showed a

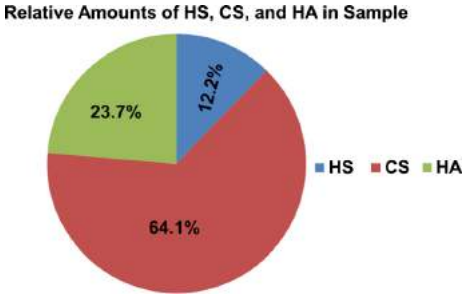


Fig. 6 Relative amounts of chondroitin sulfate, hyaluronan and heparan sulfate found in chicken gizzard

molecular mass of 120 kDa, higher that that of CS, again consistent with previous reports of HA prepared from animal sources [31]. HS was the minor GAG component in chicken muscular stomach or gizzard corresponding to 12 % of the total GAG. The HS was mainly composed of HS_{0S} and HS_{NS} with minor amounts of other disaccharide components and no $HS_{2S6S}$was present in any of the HS samples. The molecular masses of the more highly sulfated HS, fractions H2 and H3, was at the range of 21-22 kDa.The least sulfated HS fraction H1showed significant heterogeneity and a very low molecular mass of <10 kDa, suggesting that this fraction of HS might have been degraded possibly due the action of chicken heparinase [32].

In summary, chicken muscular stomach or gizzard represents a convenient source of GAGs from a muscle tissue. The GAGs isolated while showing some interesting characteristics are representative of previously reported GAG structures. Chicken gizzards represent a readily available and inexpensive tissue for GAG preparation and further studies.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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