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Glycosaminoglycans from bovine eye vitreous humour and interaction with collagen type II

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Abstract

Glycosaminoglycans (GAGs) play an important role in stabilizing the gel state of eye vitreous humour. In this study, the composition of GAGs present in bovine eye vitreous was characterized through disaccharide analysis by liquid chromatography-mass spectrometry. The interaction of GAGs with collagen type II was assessed using surface plasmon resonance (SPR). The percentage of hyaluronic acid (HA), chondroitin sulfate (CS) and heparan sulfate (HS), of total GAG, were 96.2%, 3.5% and 0.3%, respectively. The disaccharide composition of CS consisted of 4S (49%), 0S (38%) 6S (12%), 2S6S (1.5%) and 2S4S (0.3%). The disaccharide composition of HS consisted of 0S (80%), NS2S (7%), NS (7%), 6S (4%), NS6S (2%), and Tris, 2S and 4S6S (each at 0.1%). The average molecular weights of CS and HS were 148 kDa and 204 kDa, respectively. SPR reveals that collagen type II binds to heparin (primarily composed of Tris) with a binding affinity (K_D) of 755 nM and interacts with other GAGs, including CSB and CSE. Both bovine vitreous CS and HS interact with collagen type II, with vitreous HS showing a higher binding affinity.

Keywords

glycosaminoglycan; composition; interaction; collagen type II; bovine vitreous

1. Introduction

The vitreous humor of the eye is a highly-hydrated (99% water) tissue that is in contact with the retina and helps to keep it in place by pressing it against the choroid. The vitreous hydrogel maintains clarity by acting as a barrier limiting cellular invasion and also acts to protect sensitive tissue such as the crystalline lens from oxidative damage [1–4].

Biochemical analysis reveal that vitreous consists of a fibrillar component, comprised of

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collagen and glycosaminoglycans (GAGs), chiefly hyaluronan (HA) with much lower concentrations of chondroitin sulfate (CS) and heparan sulfate (HS). The core of each fibril is composed of type V/XI collagen, which is surrounded by a sheath of fibrillar type II collagen as well as a regular arrangement of type IX collagen along the outermost surface of the fibril, HA provides a swelling pressure to the collagen network and hence the vitreous gel [5–6]. Recent research has shown that the loss of CS chains from type IX collagen of the fibril surfaces together with an increased surface exposure of type II collagen can result in collagen fibrillar aggregation. Gradual and progressive aggregation of the collagen fibrils results in a redistribution of the collagen fibrils, leaving areas devoid of collagen fibrils and thereby converted into a liquid. This pathological process can progress to posterior vitreous detachment (PVD), causing blindness [7–9]. Clarifying the chemical structure of CS and other GAGs within the vitreous can improve our understanding the interaction between GAGs and collagen, important for stabilizing the vitreous fibrillar network.

Type IX collagen and versican are reportedly the major CS containing proteoglycans in the vitreous. Structural characterization of CS has relied on the isolation of vitreous proteoglycans and the subsequent analysis of the disaccharide compositions of their GAG chains. In this way, the CS GAG chains of type IX collagen in bovine vitreous was determined to be 15–60 kDa consisting predominantly of 4-sulfated (50%) and 6-sulfated (30%) with lesser amounts of unsulfated (18%) disaccharides residues [10–11]. The CS GAG chains of vitreous type IX collagen from sheep and goat showed similar molecular weights (M_w) and disaccharides compositions [12]. The CS GAG chains of chicken vitreous type IX collagen was much larger M_w (350 kDa) and rich in 6-sulfated disaccharides (63%) with only minor amounts of 4-sulfated disaccharides (17%) [13–14]. The versican proteoglycan in human vitreous is multiply substituted with CS GAG chains of 37 kDa, in which 6-sulfated disaccharides predominate, while versican from the vitreous of pigs, sheep and goats are mainly composed of 4-sulfated disaccharide [15–17]. Although isolation of specific proteoglycans is often beneficial to understand their structure and biological functions, it can be difficult to isolate, quantify and characterize these PGs when present at very low concentrations. Kim and coworkers isolated and identified hundreds of proteins from the vitreous humor of patients with proliferative diabetic retinopathy and small idiopathic macular holes [18]. This study suggests that there are many additional proteins, other than collagen type IX and versican that might carry CS chains in bovine vitreous. Thus, in this study we utilize an approach to analyze the total CS component within the bovine vitreous to better understand its structure and function and as a baseline for the study of changes related to pathogenesis [19–21].

Vitreous is a viscoelastic hydrogel, which is believed to be maintained through the interaction between its major macromolecular components including collagen, HA, proteoglycans and glycoproteins. These interactions, while provided vitreous with its viscoelastic property are poorly understood. Research on structural interactions between collagen and proteoglycans in extracellular matrix suggest that it is generally the protein core of proteoglycans that attach to collagen fibril, while the GAGs are free to interact with other components of matrix [22–25]. GAGs can bind to many different proteins, for example, heparin, a highly sulfated type of HS, reportedly binds to collagen type V and fibronectin [26–27]. GAGs can also self-interact or interact with other GAGs (through

bridging with proteins or divalent cations), as happens in cornea where antiparallel and transient interactions between CS/dermatan sulfate (DS), keratan sulfate (KS) and CS/DS-KS allow cornea tissue to respond external stress [28]. HS proteoglycans in vitreous were first isolated from vitreous by Allen and coworkers and again was identified in chick vitreous along with other proteins like fibronectin in bovine vitreous [29]. Possibly due to the low concentration of HS in vitreous, there is little information on their functional interactions except for the suggestion that they can selectively suppress diffusion of solutes based on electrostatics [30]. Munakata and coworkers studied the interaction between collagen and GAGs using surface plasmon resonance (SPR) and found no direct interaction of HA with collagen type II and type IX, the dominant forms found in vitreous, other research, based on electron microscopy, revealed rabbit vitreous network contained thin threads of HA coating and connecting collagen fibrils, suggesting the importance of GAG-collagen interactions [31, 20]. Further investigation of the molecular interaction of GAGs and collagen are called for to better understand the stabilization of vitreous network and for the design of vitreous substitutes.

In the current study, we employed bovine eye as a model to investigate the total GAG composition of vitreous humor using enzymatic digestion followed by liquid chromatography–mass spectrometry (LC-MS). Through the use of specific polysaccharide lyases, CS and HS were prepared from bovine vitreous. SPR were used to study the kinetics of interaction between heparin (HP) a highly sulfated type of HS, and collagen type II and competition between immobilized heparin and soluble CS and HS, prepared from bovine vitreous, was used to determine the preferences of binding of collagen type II to GAGs.

2. Materials and methods

2.1 Materials

Twenty bovine eyes were purchased from Bioreclamation IVT (Westbury, NY). Collagen type II from chicken sternal cartilage, HA from Rooster Comb were from Sigma-Aldrich (St. Louis, MO). Porcine intestinal HP (16 kDa) and porcine intestinal HS (12 kDa) were purchased from Celsus Laboratories (Cincinnati, OH). Porcine rib cartilage CS-type A (CSA) (20 kDa) porcine intestinal CS-type B (CSB) (30 kDa) and shark cartilage CS-type C (CSC) (20 kDa) were purchased from Sigma (St. Louis, MO). Whale cartilage CS-type D (CSD) (20 kDa) and squid cartilage CS-type E (CSE) (20 kDa) were purchased from Seikagaku (Tokyo, Japan). KS (14.3 kDa) was isolated from bovine cornea [32]. Unsaturated disaccharide standards of CS ($0S_{CS}$: UA-GalNAc, $4S_{CS}$: UA-GalNAc4S, $6S_{CS}$: UA-GalNAc6S, $2S_{CS}$: UA2S-GalNAc, $2S4S_{CS}$: UA2S-GalNAc4S, $2S6S_{CS}$: UA2S-GalNAc6S, $4S6S_{CS}$: UA-GalNAc4S6S, and $TriS_{CS}$: UA2S-GalNAc4S6S), unsaturated disaccharide standards of HS ($0S_{HS}$: UA-GlcNAc, NS_{HS} : UA-GlcNS, $6S_{HS}$: UA-GlcNAc6S, $2S_{HS}$: UA2S-GlcNAc, $2SNS_{HS}$: UA2S-GlcNS, $NS6S_{HS}$: UA-GlcNS6S, $2S6S_{HS}$: UA2S-GlcNAc6S, and $TriS_{HS}$: UA2S-GlcNS6S), unsaturated disaccharide standard of HA ($0S_{HA}$: UA-GlcNAc), and saturated disaccharide standard of KS ($KS2S$: Gal6S β 1 \rightarrow 4GlcNAc6S, $KS1S$: Gal β 1 \rightarrow 4GlcNAc6S) where UA is 4-deoxy- α -L-threo-hex-4-enopyranosyluronic acid, S is sulfo, and Ac is acetyl, were from iduron (UK). Actinase E was obtained from Kaken Biochemicals (Tokyo, Japan). Chondroitin lyase AC,

Chondroitin lyase ABC from *Proteus vulgaris* and recombinant *Flavobacterium* heparinase I, II, and III were expressed in *E. coli* in our laboratory. Keratan sulfate degrading enzyme, keratinase II, from *Bacillus circulans* was recombinantly expressed in *E. coli* [33]. Hyaluronidase from bovine testes (a hydrolase) and hyaluronidase from *Streptomyces hyalurolyticus* (a lyase) were purchased from Sigma-Aldrich (St. Louis, MO). 2-Aminoacridone (AMAC), sodium cyanoborohydride (NaCNBH₃) and 3-[(3cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were obtained from Sigma-Aldrich (St. Louis, MO). HBS-EP (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20) buffer, streptavidin (SA) sensor chips were from GE healthcare (Pittsburgh, PA). Amine-PEG3-biotin was purchased from Thermo Fisher Scientific (Waltham, MA). All other chemicals were of high-performance liquid chromatography (HPLC) grade.

2.2 Extraction of GAGs from bovine vitreous

The vitreous humor were removed by making incision through the eye posterior to the vitreous base and scooping out the vitreous gel from the posterior cup. Care was taken to avoid contamination with aqueous humor, retina and other ocular tissues. The obtained vitreous was subjected to proteolysis by adding actinase E to 5 mg/mL at 55 °C for 48 h. After dialysis in distilled water for 2 days, the solution was concentrated using a molecular weight cut-off (MWCO) 3 kDa ultracentrifuge filter and freeze-dried. The dried material was then dissolved in 8 M urea / 2 wt % CHAPS, applied to Maxi Q H spin columns (Sartoriou Stedim Biotech, NY) that were pre-equilibrated with 20 mL 8 M urea/2 wt % CHAPS. The column was then washed with 20 mL 8 M urea/2 wt % CHAPS and 20 mL 0.2 M NaCl, total GAGs were eluted with 4 mL 16% NaCl for 3 times, desalted using Amicon Ultra-4 Centrifugation filter with MWCO of 3 kDa and lyophilized.

2.3 Preparation of CS from bovine vitreous

Hyaluronidase from bovine testes digestion of HA was carried out according to the manufacture's protocol with some modification. In brief, HAase (750~1500 units per mg) was dissolved in enzyme diluent buffer (20 mM phosphate buffer with 77 mM NaCl and 0.01% bovine serum albumin, pH 7.0) to obtain a working solution of 120 units/mL, HA was dissolved in 300 mM sodium phosphate (pH 5.35) to a final concentration of 0.3 mg/mL. The same volume of HAase and GAGs were mixed gently and incubated at 37 °C for 24 h. Alternatively, double amount of HAase were added to HA for double the incubation time (48 h). Solution was then applied to Corning Spin-X UF Concentrator with MWCO 5 kDa and Amicon Ultra-0.5 Centrifugation Filter with MWCO of 10 kDa, respectively, the HA oligosaccharides in filtrates were further desalted through a 3 kDa MWCO centrifugation filter and detected by the Thermo Scientific LTQ ORBITRAP XL.

The isolated total GAGs were dissolved in digestion buffer (100 mM ammonium acetate containing 10 mM CaCl₂, pH 7.5) and treated with a mixture of heparin lyase I, II, III (10 milli-units of each) at 37 °C overnight. The solution was then applied 3 kDa MWCO centrifugation filter to remove salts and HS disaccharides, the retented sample was treated with HAase from *Streptomyces hyalurolyticus* to degrade HA, applied to MWCO 10 kDa centrifuge filter to remove HA fragments and further purified though Mini H Q spin column

to get bovine vitreous CS. The purity of prepared CS was checked through disaccharide compositional analysis by LC-MS.

2.4 Preparation of HS from bovine vitreous

The total isolated GAGs, containing a mixture of HA, CS and HS, were dissolved in digestion buffer (100 mM NH₄OAc containing 10 mM CaCl₂, pH 7.5). Chondroitin lyase ABC (20 mU in 25 mM Tris, 500 mM NaCl, 300 mM imidazole buffer, pH 7.4) was added and the solution was incubated at 37 °C overnight. After the digestion was complete, the samples were filtered through a 3 kDa MWCO centrifugation filter to remove disaccharides of CS and HA, washed twice with distilled water, the retentate was freeze-dried. The dried sample were dissolved in 8 M urea and 2 wt % CHAPS, applied to MINI Q H spin columns. HS were eluted with 16% NaCl followed by desalting using 3 kDa MWCO centrifugation filter. Similarly, the purity of prepared HS was checked through disaccharide compositional analysis by LC-MS.

2.5 Compositional analysis of disaccharides by Liquid Chromatography and Mass Spectrometry (LC-MS)

The GAG substrate (10 µg/µL) was treated with a mixture of heparin lyase I, II, III (10 mU of each) or chondroitin lyase ABC (10 mU) and incubated at 37 °C overnight to prepare HS disaccharides or CS/HA disaccharides. After a full digestion, the products were applied to MWCO 3 kDa ultracentrifuge filter, washed twice with distilled water, and the filtrate was collected, freeze-dried and AMAC-labeled by adding 10 µL of 0.1 M AMAC in dimethylsulfoxide/acetic acid (17/3, v/v) incubating at room temperature for 10 min, and adding 10 µL of 1 M aqueous NaBH₃CN incubating at 45 °C for 1 h. After centrifugation for 10 min at 14000 × *g*, supernatant was recovered for HPLC-MS/MS. Mixture containing 9 CS/HA disaccharide standards, 8 HS disaccharide standards and 17 CS/HA/HS disaccharide standards were similarly AMAC-labeled, respectively and used as external standards.

HPLC was performed on an Agilent 1200 LC system equipped with a 6300 ion trap and a binary pump. The LC column was an Agilent Poroshell 120 EC-C18 (2.7 µm, 3.0 × 150 mm) column. The column temperature was 45 °C. The flow rate was 150 µL/min. The mobile phases were 50 mM ammonium acetate (A) and methanol (B) with gradient of 5 to 30% B from 0 to 20 min, 30 to 50% B from 20 to 30 min, 100% B from 30 to 40 min, and 5% B from 40 to 50 min. The MS parameters were electrospray in negative-ionization mode with a skimmer potential of -40.0 V, a capillary exit of -40.0 V, and a source temperature of 350 °C. The mass range of the spectrum was *m/z* 300–900. Nitrogen (8 L/min, 40 psi) was used as the drying and nebulizing gas.

Similarly, the GAGs substrate (80 µg/µL) was treated with keratan sulfate degrading enzyme, keratinase II (800 mU), followed by ultrafiltration and AMAC labeling of KS disaccharides and detection by HPLC-MS as described above. Difference was that an Agilent Poroshell 120 ECC18 (2.7 µm, 3.0 × 50 mm) column was used and the gradient of mobile phase was 0–10 min, 5–45% B; 10–10.2 min, 45–100% B; 10.2–14 min, 100% B; 14–22 min, 100–5% B.

2.6 ESI-LTQ-Orbitrap-FT-MS analysis of HA oligosaccharides

HA oligosaccharides produced by hyaluronidase from bovine testes digestion of HA were analyzed in the negative-ion mode by electrospray ionization FT-MS on a Thermo Scientific LTQ Orbitrap XL FT mass spectrometer with a standard, factory-installed ion source (Thermo Fisher Scientific, San-Jose, CA) as reported [34]. External calibration of mass spectra produced a mass accuracy of < 3 ppm. Mobile phase consisting of 0.1% formic acid in 50% aqueous methanol was delivered by an Agilent 1200 nano-LC pump at a flow rate of 50 μ L/min. Desalted HA oligosaccharides (3 μ L) were dissolved in the mobile phase and introduced by direct infusion through an Agilent 1200 autosampler. Mass spectra were acquired at a resolution between 30,000 and 60,000 under the following conditions: spray voltage 3 kV, capillary temperature 200°C, capillary voltage -15 V, tube lens -100 V, the sheath and auxiliary gas flow rates were set to 20 and 5 units respectively. Product-ions were assigned using accurate mass measurement and GlycoWorkbench [35].

2.7 Interaction of bovine vitreous GAGs with collagen type II

2.7.1 Preparation of a heparin sensor chip—The streptavidin-biotin linkage was selected to attach heparin to the chip surface. Heparin was biotinylated through reaction of the free amino groups in heparin with sulfo-*N*-hydroxysuccinimide long-chain biotin (Pierce, Rockford, IL) according to a published procedure [36]. Biotinylated heparin was then immobilized to streptavidin (SA) sensor chip (GE Healthcare, Uppsala, Sweden). Briefly, a 20 μ L of the biotinylated heparin (0.1 mg/mL) in HBS-EP running buffer was injected over the second flow-cell (FC2) of a SA sensor chip at a flow-rate of 10 μ L/min. Successful immobilization of heparin was confirmed by an approximately 100 resonance units (RU) increase in the sensor chip. A control FC was prepared with a 1 min injection with saturated biotin in HBS-EP buffer. SPR measurements were performed using a BIAcore 3000 SPR instrument.

2.7.2 SPR binding kinetics of collagen type II-heparin interactions—SPR measurements were performed on a BIAcore 3000 system operated with BIAcore 3000 Control from GE healthcare. The binding kinetics of the collagen type II interactions over the heparin sensor chip were assayed using HBS-EP buffer at 25 °C. Prior to assay, collagen type II was dissolved in 50 mM acetic acid at a concentration of 5 mg/mL at 4 °C overnight, and the resulting solution was diluted by adding HBS-EP buffer to a concentration of 1000 nM. Two-fold serial dilutions of collagen type II were injected over the heparin sensor chip at a flow-rate of 30 μ L/min for 3 min. After that, 90 μ L HBS-EP buffer passed over the sensor chip surface for dissociation followed by washing with 30 μ L 2 M NaCl for regeneration. SPR experiments at each concentration were performed in triplicate. RU was monitored as a function of time (sensorgram) at 25 °C. The binding sensorgrams were pooled, trimmed, double referenced, and experimental fit to different kinetic models using BIAevaluation software v4.1.

2.7.3 SPR solution competition study of different GAGs—For testing of inhibition of other GAGs to the collagen type II/heparin interaction, 100 μ L collagen type II at 1000 nM was pre-mixed with 100 μ L 2000 nM of GAG and injected over the heparin chip at a

flow-rate of 30 ml/min. After each run, a dissociation period and regeneration protocol was performed as described above.

3 Results

3.1 Disaccharide composition of GAGs from bovine vitreous

A three step procedure was used, including Actinase E digestion, strong anion exchange (SAX) chromatography and ultracentrifuge filtration, to isolate GAGs. The total GAG isolated from bovine vitreous was then determined by carbazole method. The GAG content of the freeze-dried vitreous (0.11g per bovine eye) was 2.3%. The total GAGs could be separated into CS/HA by selective treatment with heparin lyases, which converts heparin (HP)/HS into disaccharide that can be removed from CS/HA through MWCO 3 kDa centrifuge filter and further detected for HP/HS compositional analysis. Similarly, HP/HS could be prepared by treatment with chondroitin ABC lyase, which degrades the CS/HA into disaccharides that could be removed and detected for CS/HA compositional analysis. Based on the compositional disaccharide analysis, HA was found to be the dominant GAG in bovine vitreous with the content of HA, CS/DS and HS in total GAGs to be 96.2%, 3.5% and 0.3%, respectively.

Bovine vitreous CS consisted of 2S4S, 2S6S, 4S, 6S and 0S with 4S (48.8%), 0S (37.6%) and 6S (11.9%) as the main compositional disaccharides and 2S6S (1.5%) and 2S4S (0.3%) as minor ones. To check if dermatan sulfate (DS or CSB) existed in bovine vitreous, same amount of GAGs were treated with CS_{ABC} to degrade CS including DS, or CS_{ACII} to degrade CS instead of DS, respectively. DS is a linear copolymer of N-acetylgalactosamine (GalNAc), iduronic acid (IdoA) and glucuronic acid (GlcA) by β 1,4 or 1,3 linkages with *O*-sulfo groups most commonly found on the 4-position of GalNAc residues and occasionally on the 6-position of GalNAc and the 2-position of IdoA. The presence of IdoA in DS distinguishes it from CS-A (4-*O*-sulfo) and CS-C (6-*O*-sulfo) [37]. The treatment of DS with CS_{ABC} may produce disaccharide like 2S4S, 2S6S, 4S, 6S and 0S that will result in different content of disaccharide as compared with that produced from the sample treated with CS_{ACII}. However, the CS/DS/HA disaccharide compositions of both samples were nearly the same, suggesting the absence of DS in bovine vitreous (data not shown). Keratan sulfate (KS) has been found in eye tissues like cornea. In this study, one fourth of total GAGs from one bovine vitreous were treated with keratanase II and the product was analysis for KS disaccharide [38]. Two peaks corresponding to standard Gal6S β 1 \rightarrow 4GlcNAc6S and Gal β 1 \rightarrow 4GlcNAc6S appeared (Fig. 1). However, the amount of KS was quite small and was calculated to be < 0.1% of total GAGs. The disaccharide composition of HS is presented in Table 1. The NS6S, NS2S, NS, 6S, 2S and 0S disaccharides were all detected, but the 0S (79.7%), NS2S (7.4%) and NS (7.0%) disaccharides were dominant.

3.2 Preparation of CS from bovine vitreous

Enzymatic digestion is an efficient and specific way to isolate CS/HA or HS. Treating GAGs with heparin lyase I, II, III affords intact CS and HA that can be easily isolated from the resulting HS/HP disaccharides using a MWCO 3 kDa centrifuge filter. However it is

challenging to separate CS and HA since both are sensitive to chondroitinase and HA dominates the GAGs present in bovine vitreous.

Hyaluronidase treatment can be used to remove HA. Hyaluronidase from bovine testes (bovine testicular hyaluronidase, BTH) is an endo β -acetyl-D-hexosaminidase that hydrolyzes HA at the β 1,4-N-acetylglucosaminide bonds, yielding mainly a mixture of tetrasaccharide and hexasaccharide with GlcA at the non-reducing terminus. [39] Hyaluronidase from *Streptomyces hyalurolyticus* is specific for HA and does not act on CS, cleaving at GlcNAc-(1 \rightarrow 4)GlcA, yielding tetrasaccharides and hexasaccharides with 4,5-unsaturated uronic acids at the non-reducing terminals [40]. Bovine vitreous GAGs were first digested with Heparin lyase I, II and III to remove HS, followed by digestion of hyaluronidase from *Streptomyces hyalurolyticus* to remove HA and the remaining material was then purified with Mini Q H spin column to obtain the CS component of the GAG mixture.

Standard HA was used as a control and digested with hyaluronidase from bovine testes according to the manufacturers' protocol at 37 °C for 24 h, the solutions were then applied to centrifugal filters with MWCO of 5 kDa and 10 kDa, respectively in order to remove the tetrasaccharide and hexasaccharide products of HA. Fractions that passed through the filters were desalted through 3 kDa filter and were detected by ESI-LTQ-orbitrap-FT-MS. Fig.2 shows the extracted ion chromatographs (EICs) and FTMS spectra of HA oligosaccharides passing through centrifugal filters with MWCO of 5 kDa and 10 kDa. For fractions passing through centrifugal filters with MWCO of 5 kDa, extraction of particular signals with m/z range of 387.10 to 387.12 and m/z range of 576.65 to 576.67 lead to main peak observed at 0.28 min. In the corresponding mass spectrum, the appearance of anion with $m/z = 387.11$ ($z = 2$) which could be assigned to saturated tetrasaccharide of HA (HA4), and anion with $m/z = 576.66$ ($z = 2$) which attributed to saturated hexasaccharide of HA (HA6), suggesting that centrifugal filter with MWCO 5 kDa could be used for the removal of these HA fragments. However, the difference between the EICs of fraction passing through centrifugal filters with MWCO of 5 kDa and 10 kDa was notable in that more HA6 was removed as compared with HA4 in the fraction passing through 10 kDa filters than that in fraction passing through 5 kDa filter. In addition, it has been reported that HA oligosaccharides of certain size (dp) of 10 to 12 can be somewhat resistant to *Streptomyces* hyaluronate lyase, suggesting that MWCO 10 kDa centrifugal filter was more efficient to remove HA oligosaccharides than a MWCO 5 kDa centrifugal filter [40]. MWCO 10 kDa centrifugal filter could be more powerful to remove HA fragments than MWCO 5 kDa centrifugal filter in case that HA is not completely digested to tetrasaccharide and hexasaccharides products.

The EIC of bovine vitreous GAGs after being digested with HPase I, II, III and HAase followed by purification with MWCO 10 kDa centrifugal filter and Mini Q H spin column is shown in Fig. 3. No HS was detected, the HA content in CS/HA was 17%. The content of CS0S decreased and the content of CS2S4S, CS4S and CS6S increased compared to the original composition of CS (Table 1). Employing HAase digestion followed centrifugal filtration with a MWCO 10 kDa centrifugal filter represents an efficient way to prepare CS with a reduced content of HA. PAGE analysis of the recovered CS suggests that the average molecular weight of bovine vitreous CS was 148 kDa (data not shown).

3.3 Preparation of HS from bovine vitreous

HS could be prepared through CS_{ABC} digestion of GAGs, removal of CS/HA disaccharides through MWCO 3 kDa centrifugal filter and further purification by Mini Q H spin column. The EIC of the prepared HS is shown in Fig. 3 No CS or HA was detected in the recovered HS. PAGE shows the average molecular weight of bovine vitreous HS was 204 kDa (data not shown).

3.4 Kinetic measurements of collagen type II–heparin interaction

Electrostatic interaction is believed to be the main type of interaction between various proteins and GAGs. HP is the most negatively charged GAGs. On this basis, we examined the interaction of HP with collagen type II by a real time SPR binding assay to determine binding kinetics. Collagen type II was injected over the surface of a sensor chip with immobilized heparin. Sensorgrams of collagen type II–heparin interaction are shown in Fig. 4. Sensorgrams were globally fit to calculate the apparent on (k_a) and off (k_b) rates for the binding equilibrium, using the BioEvaluation software based on a Langmuir model. The calculated constants k_a , k_b and the binding affinity (K_D) were $4.48 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, $2.62 \times 10^{-3} \text{ s}^{-1}$, and 755 nM, respectively.

3.5 Solution competition study of various GAGs and bovine vitreous CS and HS

Previous studies showed that an efficient GAG-protein interaction required GAGs with specific saccharide sequence and sulfation patterns. We then screened various GAGs for their ability to inhibit collagen type II and HP interaction. A mixture of collagen type II and various GAGs were injected over the surface of the HP-immobilized sensor chip. SPR competition sensorgrams and graphs for the GAG competition levels are presented in Fig. 5. For collagen type II, heparin produced the greatest inhibition by competing with 95% of the collagen binding to the immobilized heparin signal. Strong inhibitory activities (> 80%) were observed for CSB and CSE. Modest inhibitory activities (> 50%) were observed for HS, CSC, CSD and KS. Weak inhibitory activity was observed for CSA.

Based on the average molecular weight, 1 μM of bovine vitreous CS and HS were prepared in HBS-EP buffer, mixed with collagen type II and SPR competition assay was applied to determine the binding preference of collagen type II for them. Modest inhibitory activity (35%) was observed for bovine vitreous HS, while weak inhibitory activity (60%) was observed for bovine vitreous CS (Fig. 5).

4. Discussion

Since the content of sulfated proteoglycans is relatively low in vitreous, isolation of proteoglycans and then structural characterization of GAGs from the isolated preteoglycans can be time-consuming and affords only qualitative data. In this study, the composition of total GAGs in bovine vitreous was efficiently analyzed based on the optimized experimental condition of LC-MS. In this study, 0.8% (v/v) of vitreous (one eye) was enough to obtain the disaccharide composition of CS, HA, and HS. We found that 4S, 6S and 0S were the main compositional CS disaccharides with the content of unsulphated chondroitin (38%) much higher than the previous reports about CS disaccharide composition of versican and collagen

type IX of bovine vitreous [10–11]. One reason for the structural difference might be the different physiological condition of cow eye used in experiment in terms of age and sex because more unsulfated CS could be detected in eyes with older ages and symptom like retinal detachment [41]. The other reason was that additional CS containing proteoglycans beyond versican and collagen type IX, might be present in bovine vitreous [18]. CS prepared through hyaluronase digestion and filtration through MWCO 10 kDa filters, showed a decreased content of unsulfated CS disaccharide (5.8%), implying the low molecular weight of most of the unsulfated residue containing CS. DS was not found in bovine vitreous, which was consistent with previous studies and KS was detected in vitreous for the first time despite its very low concentration. The composition of HS was reported for the first time in the current study with OS (79.7%), NS2S (7.4%) and NS (7.0%) representing the dominant HS disaccharide components.

GAGs are generally proposed to play an important role in stabilizing the vitreous gel, however GAG-GAG and GAG-collagen interactions are not well understood. HA comprises the vast majority of GAGs within the vitreous. In solution, HA molecules intertwine at low concentration and become entangled at higher concentration [42]. HA is also a template for assembly of other extracellular macromolecules like versican. Versican has an *N*-terminal HA-binding domain and the binding to HA is stabilized by link protein [43]. Chondroitin 6-sulfate is capable of self-associating and were able to form the extended filamentous glycosaminoglycan networks which are found in vitreous, whereas chondroitin 4-sulfate is not able to form such networks, however, it may be able to form heteroduplexes with hyaluronan [44–45]. Chondroitin 6-sulfate has been shown to interact specifically with hyaluronan *in vitro* [46]. The relatively short glycosaminoglycan chains of bovine vitreous type-IX collagen are not needed to form an extended filamentous glycosaminoglycan meshwork in the hyaluronan-rich mammalian vitreous. They may provide a mechanism whereby the major collagen fibrils of bovine vitreous can interact with the hyaluronan meshwork (including chondroitin 4- and 6-sulfate) or with adjacent collagen fibrils (through chondroitin 6 sulfate duplexes) and consequently may be of key importance in the supramolecular assembly of bovine vitreous [11].

The affinity of GAGs to collagen is important for maintaining a stable network of vitreous. Usually, it is the core protein that binds on the specific locations of collagen fibril surface, while the GAGs chains was hypothesized to extend in the interfibrillar space where they interact forming antiparallel dimers [47]. By using high-resolution scanning electron microscopy, GAGs (except chondroitin 4-sulfate) were found to bind to the collagen type I fibril surface in a highly specific fashion [48]. SPR showed that CSE binds to immobilized collagen II and IX [31]. We prepared the immobilized heparin chip, and found the concentration dependent binding of collagen type II to immobilized heparin. This was in contrast with results obtained when collagen type II was immobilized onto a chip and heparin-collagen type II interactions were observed [31]. A competitive SPR binding study was undertaken based on the interaction between heparin and collagen type II. A series of GAGs were applied with collagen type II to inhibit the binding of collagen type II to heparin and the competition assays showed different abilities to inhibit this interaction with HP > CSE > CSB > KS > HS > CSC > CSD > CSA. This ordering indicates that sulfation position and GAG saccharide sequence impact their binding to collagen type II. After learning that

the interaction between collagen type II and GAGs exhibits structural specificity, we next examine the interaction between collagen type II and CS or HS derived from bovine vitreous. SPR results revealed that both CS and HS from bovine vitreous competitively inhibited the binding of collagen type II to heparin. HS exhibited stronger affinity to collagen II than did CS and this is understandable considering the composition of CS and HS. Bovine vitreous CS is mainly composed with CS4S (68%) and CS6S (19%), which are also the main compositional disaccharide of CSA and CSC, while both of which exhibited very weak affinity to collagen type II (Fig 5). Although HS comprised mainly of HS0S (80%), the presence of other sulfated disaccharides, including TriS, NS6S, NS2S, NS, 2S6S, 6S and 2S, certainly contribute to the binding to collagen type II. From this point, bovine vitreous HS took part in stabilizing the super molecular gel through interaction with collagen type II.

In summary, the composition of GAGs from bovine vitreous was clarified through disaccharide analysis by liquid chromatography-mass spectrometry. CS and HS were prepared through specific enzyme digestion. Collagen type II as the main collagen in bovine vitreous was found to have a concentration dependent binding with immobilized heparin. The strong interaction of collagen type II with CSE was also proved. Both bovine CS and HS showed an interaction with collagen type II while HS had a stronger affinity.

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References

1. Shui YB, Holekamp NM, Kramer BC, Crowley JR, Wilkins MA, Chu F, Malone PE, Mangers SJ, Hou JH, Siegfried CJ, Beebe DC. The gel state of the vitreous and ascorbate-dependent oxygen consumption: relationship to the etiology of nuclear cataracts. *Arch Ophthalmol*. 2009; 127:475–482. [PubMed: 19365028]
2. Holekamp NM. The vitreous gel: more than meets the eye. *Am J Ophthalmol*. 2010; 149:32–36. [PubMed: 19875090]
3. Hikichi T, Konno S, Trempe CL. Role of the vitreous in central retinal vein occlusion. *Retina*. 1995; 15:29–33. [PubMed: 7754244]
4. Foulds WS. Is your vitreous really necessary? The role of the vitreous in the eye with particular reference to retinal attachment, detachment and the mode of action of vitreous substitutes. *Eye (Lond)*. 1987; 1(Pt 6):641–664. [PubMed: 3331605]
5. Azuma N, Tajima S, Konomi H, Hida T, Akiya S, Uemura Y. Glycosaminoglycan and collagen distribution in the developing human vitreous. *Graefes Arch Clin Exp Ophthalmol*. 1998; 236:679–687. [PubMed: 9782429]
6. Nishikawa S, Tamai M. Ultrastructure of hyaluronic acid and collagen in the human vitreous. *Curr Eye Res*. 1996; 15(1):37–43. [PubMed: 8631202]
7. Syed Z, Stewart MW. Age-dependent vitreous separation from the macula in a clinic population. *Clin Ophthalmol*. 2016; 10:1237–1243. [PubMed: 27462138]
8. Bishop PN, Holmes DF, Kadler KE, McLeod D, Bos KJ. Age-related changes on the surface of vitreous collagen fibrils. *Invest Ophthalmol Vis Sci*. 2004; 45:1041–1046. [PubMed: 15037566]
9. Cases O, Obry A, Ben-Yacoub S, Augustin S, Joseph A, Toutirais G, Simonutti M, Christ A, Cosette P, Kozyraki R. Impaired vitreous composition and retinal pigment epithelium function in the FoxG1::LRP2 myopic mice. *Biochim Biophys Acta*. 2017; 1863:1242–1254. [PubMed: 28366874]

10. Bishop, Paul, McLeod, David, Ayad, Shirley. Extraction and characterisation of the intact form of bovine vitreous type IX collagen. *Biochem Biophys Res Commun.* 1992; 185:392–397. [PubMed: 1599476]
11. Bishop PN, Crossman MV, McLeod D, Ayad S. Extraction and characterization of the tissue forms of collagen types 11 and IX from bovine vitreous. *Biochem J.* 1994; 299:497–505. [PubMed: 8172611]
12. Skandalis SS, Theocharis DA, Noulas AV. Chondroitin sulphate proteoglycans in the vitreous gel of sheep and goat. *Biomed Chromatogr.* 2007; 21:451–457. [PubMed: 17370293]
13. Yada T, Suzuki S, Kobayashi K, Kobayashi M, Hoshino T, Horie K, Kimata K. Occurrence in chick embryo vitreous humor of a type IX collagen proteoglycan with an extraordinarily large chondroitin sulfate chain and short alpha 1 polypeptide. *J Biol Chem.* 1990; 265:6992–6999. [PubMed: 2324108]
14. Brewton RG, Wright DW, Mayne R. Structural and functional comparison of type IX collagen-proteoglycan from chicken cartilage and vitreous humor. *J Biol Chem.* 1991; 266:4752–4757. [PubMed: 2002024]
15. Reardon A, Heinegård D, McLeod D, Sheehan JK, Bishop PN. The large chondroitin sulphate proteoglycan versican in mammalian vitreous. *Matrix Biol.* 1998; 17:325–333. [PubMed: 9822199]
16. Theocharis AD, Papageorgakopoulou N, Feretis E, Theocharis DA. Occurrence and structural characterization of versican-like proteoglycan in human vitreous. *Biochimie.* 2002; 84:1237–1243. [PubMed: 12628301]
17. Noulas AV, Theocharis AD, Feretis E, Papageorgakopoulou N, Karamanos NK, Theocharis DA. Pig vitreous gel: macromolecular composition with particular reference to hyaluronan-binding proteoglycans. *Biochimie.* 2002; 84:295–302. [PubMed: 12106907]
18. Kim T, Kim SJ, Kim K, Kang UB, Lee C, Park KS, Yu HG, Kim Y. Profiling of vitreous proteomes from proliferative diabetic retinopathy and nondiabetic patients. *Proteomics.* 2007; 7:4203–4215. [PubMed: 17955474]
19. Theocharis DA, Feretis E, Papageorgakopoulou N. Glycosaminoglycans in the vitreous body of patients with retinal detachment. *Biochem Int.* 1991; 25:397–407. [PubMed: 1805784]
20. Góes RM, Nader HB, Porcionatto MA, Haddad A, Laicine EM. Chondroitin sulfate proteoglycans are structural renewable constituents of the rabbit vitreous body. *Curr Eye Res.* 2005; 30:405–41. [PubMed: 16020271]
21. Theocharis DA, Skandalis SS, Noulas AV, Papageorgakopoulou N, Theocharis AD, Karamanos NK. Hyaluronan and chondroitin sulfate proteoglycans in the supramolecular organization of the mammalian vitreous body. *Connect Tissue Res.* 2008; 49:124–128. [PubMed: 18661326]
22. Scott JE. Extracellular matrix, supramolecular organisation and shape. *J Anat.* 1995; 187:259–269. [PubMed: 759190]
23. Muller LJ, Pels E, Schurmans LR, Vrensen GF. A new three dimensional model of the organization of proteoglycans and collagen fibrils in the human corneal stroma. *Exp Eye Res.* 2004; 78:493–501. [PubMed: 15106928]
24. Schönherr E, Hausser H, Beavan L, Kresse H. Decorin-type I collagen interaction: presence of separate core protein-binding domains. *J Biol Chem.* 1995a; 270:8877–8883. [PubMed: 7721795]
25. Schönherr E, Witsch-Prehm P, Harrach B, Robenek H, Rauterberg J, Kresse H. Interaction of biglycan with type I collagen. *J Biol Chem.* 1995b; 270:2776–2783. [PubMed: 7852349]
26. Ricard-Blum S, Beraud M, Raynal N, Farndale RW, Ruggiero F. Structural requirements for heparin/heparan sulfate binding to type V collagen. *J Biol Chem.* 2006; 281:25195–204. [PubMed: 16815843]
27. Carpentier M, Denys A, Allain F, Vergoten G. Molecular docking of heparin oligosaccharides with Hep-II heparin-binding domain of fibronectin reveals an interplay between the different positions of sulfate groups. *Glycoconj J.* 2014; 31:161–169. [PubMed: 24242364]
28. Lewis PN, Pinali C, Young RD, Meek KM, Quantock AJ, Knupp C. Structural interactions between collagen and proteoglycans are elucidated by three-dimensional electron tomography of bovine cornea. *Structure.* 2010; 18:239–245. [PubMed: 20159468]

29. Allen WS, Otterbein EC, Wardi AH. Isolation and characterization of the sulfated glycosaminoglycans of the vitreous body. *Biochim Biophys Acta, Gen Subj.* 1977; 498:167–175.
30. Käs Dorf BT, Arends F, Lieleg O. Diffusion Regulation in the Vitreous Humor. *Biophys J.* 2015; 109:2171–2181. [PubMed: 26588575]
31. Munakata H, Takagaki K, Majima M, Endo M. Interaction between collagens and glycosaminoglycans investigated using a surface plasmon resonance biosensor. *Glycobiology.* 1999; 9(10):1023–1027. [PubMed: 10521538]
32. Weyers A, Yang B, Solakyildirim K, Yee V, Zhang F, Linhardt RJ. Isolation of bovine corneal keratin sulfate and its growth factor and macphogen binding. *FEBS J.* 2013; 280:2285–2293. [PubMed: 23402351]
33. Wang H, He W, Jiang P, Yu Y, Lin L, Sun X, Koffas M, Zhang F, Linhardt RJ. Construction and functional characterization of truncated versions of recombinant keratinase II from *Bacillus circulans*. *Glycoconj J.* 2017; 34:643–649.
34. Ly M, Leach FE, Laremore TN, Toida T, Amster IJ, Linhardt RJ. The proteoglycan bikunin has a defined sequence. *Nat Chem Biol.* 2011; 7:827–833. [PubMed: 21983600]
35. Domon B, Costello C. A systematic nomenclature for carbohydrate fragmentations in FABMS/MS spectra of glycoconjugates. *Glycoconj J.* 1988; 5:397–409.
36. Zhang F, Liang X, Pu D, George KI, Holland PJ, Walsh ST, Linhardt RJ. Biophysical characterization of glycosaminoglycan-IL-7 interactions using SPR. *Biochimie.* 2012; 94:242–249. [PubMed: 22085638]
37. Taylor KR, Rudisill JA, Gallo RL. Structural and sequence motifs in dermatan sulfate for promoting fibroblast growth factor-2 (FGF-2) and FGF-7 activity. *J Biol Chem.* 2005; 280:5300–5306. [PubMed: 15563459]
38. Volpi N, Maccari F, Ferrari S, Luca MD, Pellegrini G. Separation of keratan-sulfate-derived disaccharides by high-performance liquid chromatography and postcolumn derivatization with 2-cyanoacetamide and fluorimetric detection. *Anal Biochem.* 2005; 342:200–205. [PubMed: 15989926]
39. Kakizaki I, Ibori N, Kojima K, Yamaguchi M, Endo M. Mechanism for the hydrolysis of hyaluronan oligosaccharides by bovine testicular hyaluronidase. *FEBS J.* 2010; 277:1776–1786. [PubMed: 20193045]
40. Park Y, Cho S, Linhardt RJ. Exploration of the action pattern of *Streptomyces* hyaluronate lyase using high-resolution capillary electrophoresis. *Biochim Biophys Acta.* 1997; 1337:217–226. [PubMed: 9048898]
41. Theocharis DA, Feretis E, Papageorgacopoulou N. Glycosaminoglycans in the vitreous body of patients with retinal detachment. *Biochem Int.* 1991; 25:397–407. [PubMed: 1805784]
42. Terbojevich M, Cosani A, Palumbo M, Pregolato F. Structural properties of hyaluronic acid in moderately concentrated solutions. *Carbohydr Res.* 1986; 149:363–377. [PubMed: 3756949]
43. Theocharis DA, Skandalis SS, Noulas AV, Papageorgakopoulou N, Theocharis AD, Karamanos NK. Hyaluronan and Chondroitin Sulfate Proteoglycans in the Supramolecular Organization of the Mammalian Vitreous Body. *Connect Tissue Res.* 2008; 49:124–128. [PubMed: 18661326]
44. Scott JE, Chen Y, Brass A. Secondary and tertiary structures involving chondroitin and chondroitin sulphates in solution, investigated by rotary shadowing/electron microscopy and computer simulation. *Eur J Biochem.* 1992; 209:675–680. [PubMed: 1425674]
45. Brewton RG, Mayne R. Mammalian vitreous humor contains networks of hyaluronan molecules: electron microscopic analysis using the hyaluronan-binding region (G1) of aggrecan and link protein. *Exp Cell Res.* 1992; 198:237–249. [PubMed: 1729132]
46. Turley EA, Roth S. Interactions between the carbohydrate chains of hyaluronate and chondroitin sulphate. *Nature.* 1980; 283:268–271. [PubMed: 6243396]
47. Iozzo RV. The biology of the small leucine-rich proteoglycans. Functional network of interactive proteins. *J Biol Chem.* 1999; 274:18843–18846. [PubMed: 10383378]
48. Raspanti M, Viola M, Forlino A, Tenni R, Gruppi C, Tira ME. Glycosaminoglycans show a specific periodic interaction with type I collagen fibrils. *J Struct Biol.* 2008; 164:134–139. [PubMed: 18664384]

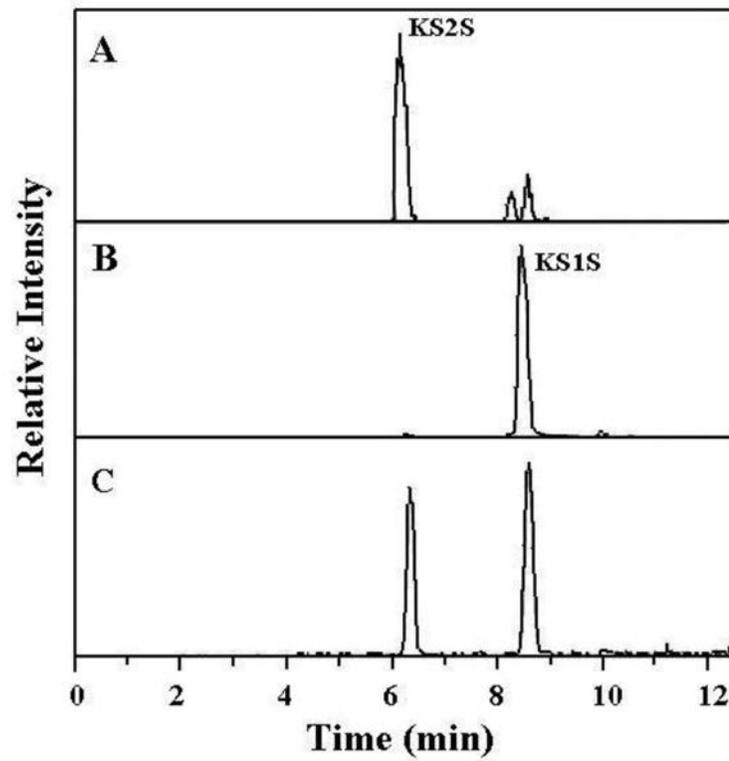


Fig. 1. Extracted ion chromatograms (EICs) of AMAC-labeled disaccharides of keratan sulfate from bovine vitreous. (A) KS2S disaccharide standard (Gal6S β 1 \rightarrow 4GlcNAc6S). (B) KS1S disaccharide standard (Gal β 1 \rightarrow 4GlcNAc6S). (C) keratan sulfate from bovine vitreous.

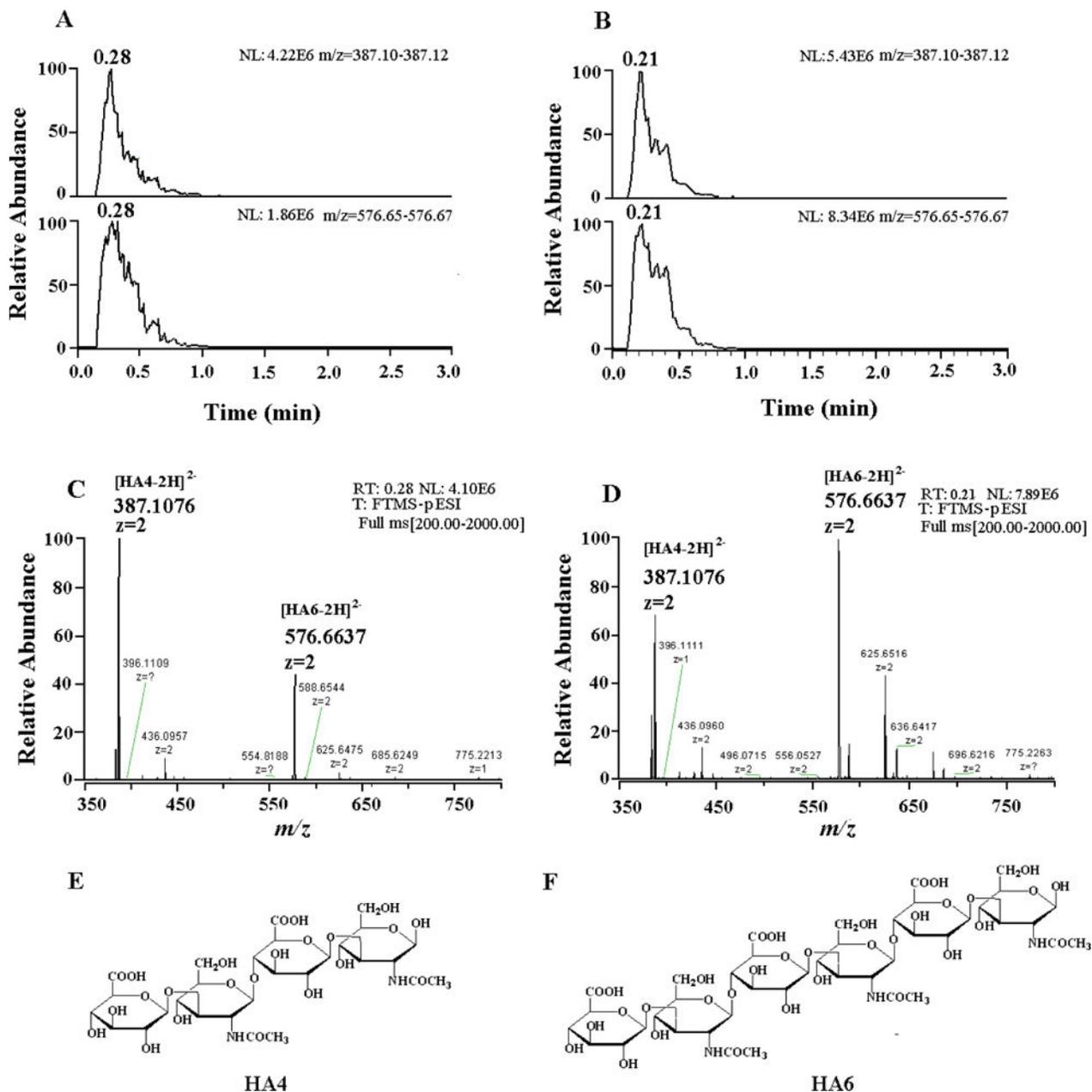


Fig. 2. EICs and Mass spectra (MS) spectra of HA fragments digested by hyaluronidase from bovine testes going through MWCO 5 kDa centrifuge filter and MWCO 10 kDa centrifuge filter. (A) EICs of HA fragments going through MWCO 5 kDa centrifuge filter with the mass range of 387.10 to 387.12 and 576.65 to 576.68, respectively. (B) EICs of HA fragments going through MWCO 10 kDa centrifuge filter with the mass range of 387.10 to 387.12 and 576.65 to 576.68, respectively. (C) MS of peak associated with retention time of 0.28 min in (A). (D) MS of peak associated with retention time of 0.21 min in (B). (E) and (F) illustrate the chemical structure of HA4 and HA6.

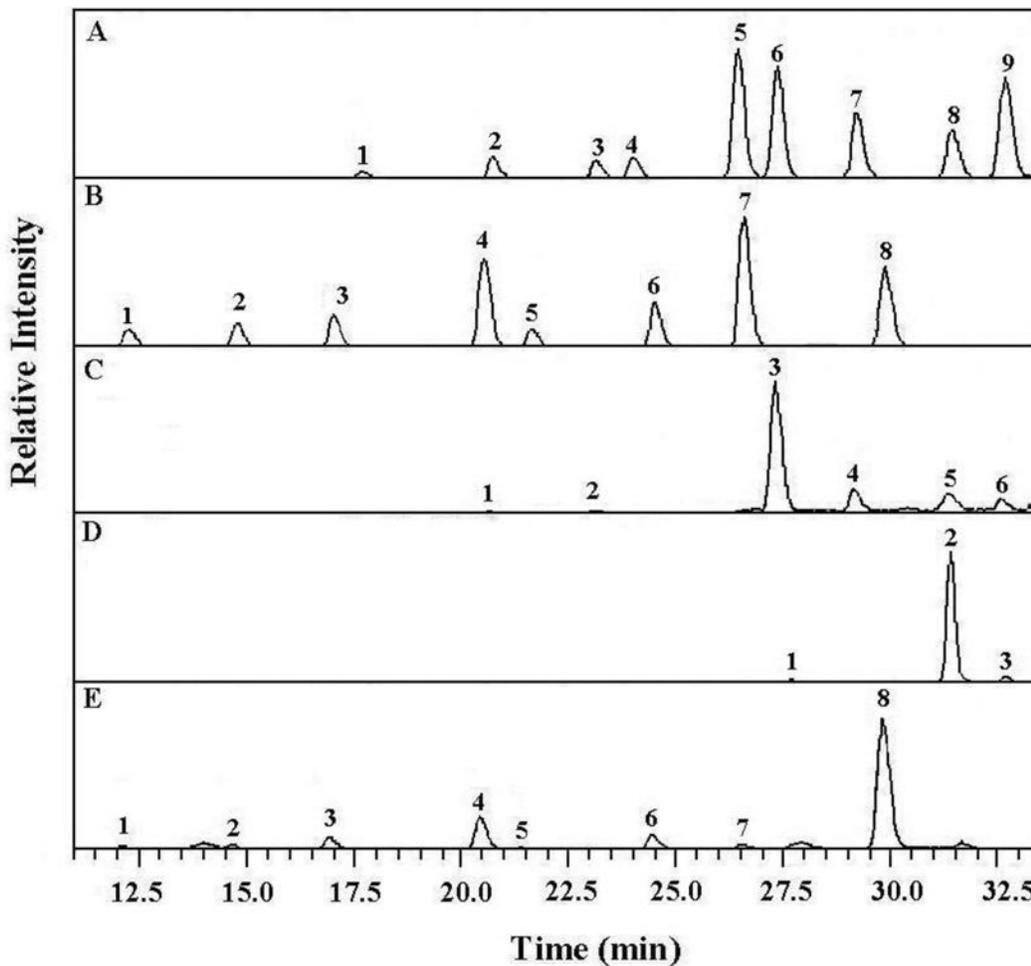


Fig. 3. EICs of AMAC-labeled disaccharides of prepared CS and HS from bovine vitreous. (A) AMAC-labeled 9 CS/HA disaccharides standard: (1) TriS_{CS}, (2) 2S4S_{CS}, (3) 2S6S_{CS}, (4) 4S6S_{CS}, (5) 2S_{CS}, (6) 4S_{CS}, (7) 6S_{CS}, (8) 0S_{HA}, and (9) 0S_{CS}. (B) 8 HS disaccharides standard: (1) TriS_{HS}, (2) NS6S_{HS}, (3) NS2S_{HS}, (4) NS_{HS}, (5) 2S6S_{HS}, (6) 6S_{HS}, (7) 2S_{HS}, (8) 0S_{HS}. (C) CS prepared from bovine vitreous: (1) 2S4S_{CS}, (2) 2S6S_{CS}, (3) 4S_{CS}, (4) 6S_{CS}, (5) 0S_{HA}, and (6) 0S_{CS}. (D) AMAC-labeled disaccharides from bovine vitreous GAGs treated with CS_{ABC}: (1) 4S_{CS}, (2) 0S_{HA}, and (3) 0S_{CS}. (E) HS prepared from bovine vitreous: (1) TriS_{HS}, (2) NS6S_{HS}, (3) NS2S_{HS}, (4) NS_{HS}, (5) 2S6S_{HS}, (6) 6S_{HS}, (7) 2S_{HS}, (8) 0S_{HS}.

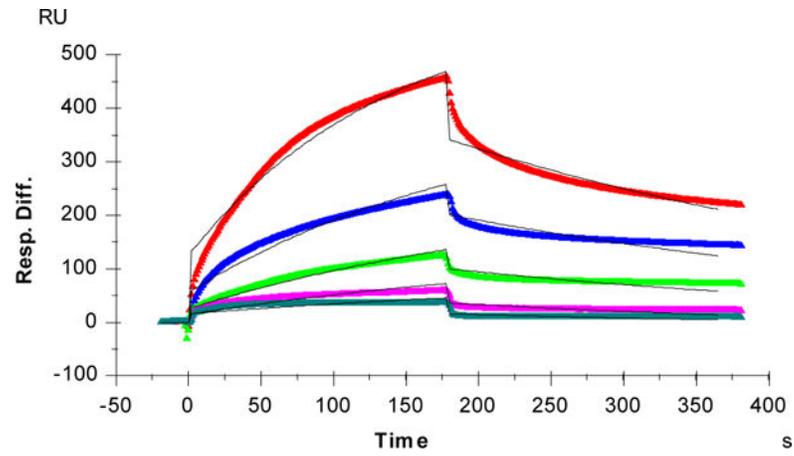


Fig. 4. SPR sensorgrams of collagen II-heparin interaction. The concentration of collagen II (from top to bottom) were 1000, 500, 250, 125, 63 nM, respectively. The black fitting curves use models from BIAevaluation 4.1.

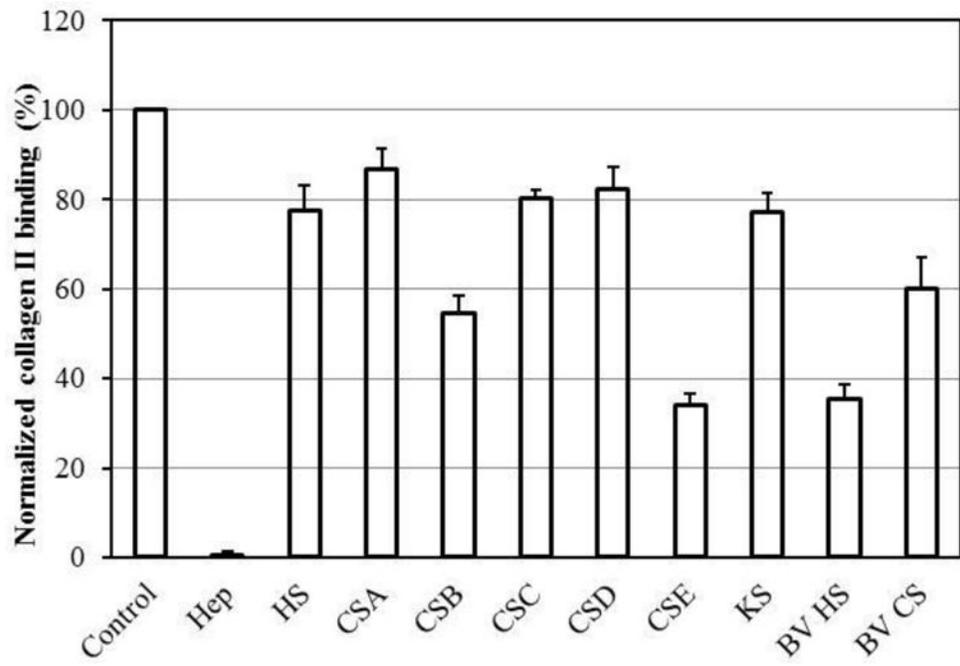


Fig. 5. Inhibition of binding of collagen II to heparin chip by various natural GAGs, CS and HS from bovine vitreous. The concentration of collagen II and GAGs in solution were both 500 nM and 1000 nM.

Table 1

Disaccharide composition of GAGs prepared from bovine eye vitreous.

		Compositional disaccharides, wt%							
CS	TriS	2S4S	2S6S	4S6S	2S	4S	6S	0S	
	ND	0.81±0.41	6.85±0.57	ND	ND	67.25±1.10	19.24±0.44	5.85±0.12	
HS	TriS	NS6S	NS2S	NS	2S6S	6S	2S	0S	
	0.13±0.11	1.85±1.72	7.39±6.59	6.97±4.39	0.09±0.11	3.79±4.26	0.10±0.12	79.68±16.66	

ND: not detected.