Characteristics of glycosaminoglycans in chicken eggshells and the influence of disaccharide composition on eggshell properties

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ABSTRACT Glycosaminoglycans (GAG) are linear, highly negatively charged polysaccharides that may perform an important role in biomineralization. GAG were isolated from chicken eggshell membranes and calcified shells. Disaccharide compositional analysis was performed using liquid chromatography-mass spectrometry. All 4 groups of GAG — hyaluronan (HA), keratan sulfate (KS), chondroitin sulfate (CS), and heparan sulfate (HS) — were detected in shell membranes and in calcified shells. HA was the most plentiful GAG in shell membranes, and CS was the most abundant in calcified shells. The CS present, in both membranes and calcified shells, consisted primarily of $6S_{CS\text{-}C}$, $4S_{CS\text{-}A}$, and $0S_{CS\text{-}0}$ disaccharides. Neither $4S6S_{CS\text{-}E}$ nor $2S_{CS}$ was detectable in shell components. Small amounts of $2S4S_{CS\text{-}B}$ were detected in membranes and $TriS_{CS}$, and $2S4S_{CS\text{-}B}$ and $2S6S_{CS\text{-}D}$ were detected in calcified shells. HS in calcified shells contained all disaccharides except for 2S6S. In shell membranes, HS contained primarily NS and 0S as well as small amounts of TriS, NS2S, $NS6S_{HS}$, and 6S, but neither 2S6S nor 2S was detectable. The disaccharide composition of membrane CS, as well as membrane and calcified shell HS, were very similar in all eggshells. In contrast, the composition of calcified shell CS disaccharides was highly variable. In membranes, both HA and KS content showed a correlation with egg shape index. The $4S_{CS\text{-}A}$ content correlated with eggshell strength, and $0S_{CS\text{-}0}$ correlated with eggshell strength and calcified shell thickness. HS content and its disaccharide composition showed no apparent correlation to properties of calcified shells. In calcified shells, only HS 6S correlated with egg shape index. This study suggests that GAG content and disaccharide composition of shell membranes might impact the quality of chicken eggshells.

Key words: eggshell quality, glycosaminoglycans, keratan sulfate, chondroitin sulfate, heparan sulfate

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INTRODUCTION

Chicken eggshell is an economic trait of importance in the chicken industry. The chicken eggshell is a calcitic porous container that serves to prevent egg desiccation, to protect the egg against mechanical damage and microbial ingression, to regulate gas exchange and moisture loss for expected hatchability, and to provide calcium for embryonic skeletogenesis (Mann et al., 2006).

An eggshell consists of the innermost eggshell membranes, a calcified layer, and the outermost cuticle (Arias et al., 1993). Eggshell membranes are a pair of fibrous layers in which highly cross-linked type I, type V, and type X collagens are arranged in core fibers that are coated with a highly glycosylated fuzzy mantle (Simons, 1971; Arias et al., 1991; Carrino et al., 1996). Ultrastructurally, the calcified layer contains a mammillary region and palisade region. External to the shell membranes are the mammillary knobs (also named calcium reserve assemblies), which are the sites for crystal nucleation and the initial phases of calcium deposition (Stemberger et al., 1977). The palisade region lies external to the mammillary layer; it is the major part of the whole shell (responsible for about 65 to 70% of the whole shell thickness) and forms the major protective barrier of the egg (Lakshminarayanan et al., 2002). Finally, the outermost and thinnest cuticle is composed of glycoproteins and hydroxyapatite crystals, and is believed to serve as a defense against microorganism ingress and for the pigmenting of colored eggs (Fraser et al., 1999; Bain et al., 2013).

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In addition to the membranes' organic extracellular matrix, the calcified shell is also a biological composite, consisting of approximately 2.5% organic matrix and approximately 95% calcitic crystals (Mann and Mann, 2013). Previous reports show that the organic matter in the shell membrane and calcified eggshell are composed of diverse proteins, proteoglycans, glycoproteins, and even small amounts of lipids (Burley and Vadehra, 1989). The organic matrix in the calcified shell is thought to have a crucial role in the ultrastructure and the biomechanical properties of the eggshell by controlling crystal nucleation, crystal growth, and morphology (Mann et al., 2002). Without the organic matrix, the crystal structure would be too brittle to keep its form.

Proteoglycans (**PG**) are biological molecules composed of a specific core protein substituted with one or more covalently linked glycosaminoglycan chains. Glycosaminoglycans (**GAG**) are linear, highly negative charged polysaccharides composed of a variable number of repeating disaccharide units. Based on the structure of their repeating disaccharides, GAG can be classified into 4 groups: hyaluronan (**HA**), chondroitin sulfate (**CS**), keratan sulfate (**KS**), and heparan sulfate (**HS**). The disaccharide repeating units of HA, CS, HS, and KS are comprised of hexosamine residue, galactosamine (**GalN**), or glucosamine (**GlcN**); for HA, CS, and HS residue, uronic acid D-glucoronic acid (**GlcA**) or L-iduronic acid (**IdoA**); and for KS, galactose (**Gal**). HA, the simplest GAG, contains neither sulfo group, nor is it attached to a core protein, and it has the structure →3) β-GlcNAc (1→4) β-GlcA (1→ (where Ac is acetyl). HS/Hp is an *O*-sulfo or *N*-sulfo group substituted GAG with repeating units of →4) α-GlcNAc or α-GlcNS (1→4) β-GlcA or α-IdoA (1→ (where S is sulfo). CS is an *O*-sulfo group substituted GAG with →3) β-GalNAc (1→4) β-GlcA or α-IdoA (1→ repeating units (Capila and Linhardt, 2002). KS is an *O*-sulfo group containing GAG with a backbone structure of →4) β-GlcNAc (1→3) β-Gal (1→ (Schaefer and Schaefer, 2010). Since GAG are acidic macromolecules with plenty of carboxyl and/or sulfo groups, proteoglycans may have the potential to function in the process of biomineralization.

Chicken eggshell contains diverse GAG, not only membranes, but also calcified shell contains all 4 groups of GAG (Carrino et al., 1997; Fernandez et al., 1997; Nakano et al., 2001; Liu et al., 2014). In a calcified eggshell, immunostaining shows that KS distributes to different regions of the eggshell, but prominently in the mammillary region and CS type-B (dermatan sulfate) is very intense in the palisade region (Carrino et al., 1996; Fernandez et al., 2001). The KS in the mammillary region is thought to perform a role in the nucleation of the first calcite crystals (Arias et al., 1992; Fernandez et al., 2001), and CS type-B is thought to have a role in the growth and orientation of the later forming crystals of the chicken eggshell (Fernandez et al., 1997, 2001). The *in vitro* evidence shows that a CS type-B proteoglycan can affect calcium carbonate crystal formation in a concentration-dependent manner (Carrino et al., 1996).

Colorimetric evidence shows that the GAG in calcified eggshell can influence eggshell morphology (shell mass per area), while the GAG in shell membranes are associated with shell strength (Ha et al., 2007). Uronic acid is a constituent sugar of all GAG except KS. There is a significant correlation between the uronic acid content in calcified shell and the shell strength (Bronsch and Diamantstein, 1965), while there is only a weak correlation between the uronic acid content in shell membranes and the shell strength (Ha et al., 2007). Furthermore, galactose is a constituent of KS, and the galactose content in eggshell membranes shows a correlation with eggshell strength.

We previously probed into the occurrence of various disaccharides of each group of GAG using LC-MS in mixed shell membranes and calcified shells from several chicken eggs, (Liu et al., 2014). In the present study, we further probe into the population characteristics of various GAG disaccharides in chicken eggshells and correlate these to eggshell properties. The results of this study should help us understand the influence of GAG fine structure on eggshell properties and broaden the potential candidates in marker-assisted selection for the improvement of chicken eggshell quality.

MATERIALS AND METHODS

Materials

Twenty-eight experimental eggs, which were laid by 48-week-old caged Hy-line Brown commercial layers, were chosen from original specimens according to the following rules. To minimize the size difference, egg weight of eggs ranged 56 to 66 g; to minimize the shape difference, shape index (length/width) of eggs ranged from 1.25 to 1.34; to minimize the thickness heterogeneity in the same calcified eggshell, at least 6 samples were determined in each area, such as blunt, sharp, and equator areas; furthermore, the maximum thickness difference in each area should be less than 0.03 mm, and the maximum difference should be less than 0.03 mm among the average thickness of the above 3 areas.

Unsaturated disaccharide standards of CS ($0S_{CS\text{-}0}$: ΔUA-GalNAc; $4S_{CS\text{-}A}$: ΔUA-GalNAc4S; $6S_{CS\text{-}C}$: ΔUA-GalNAc6S; $2S_{CS}$: ΔUA2S-GalNAc; $2S4S_{CS\text{-}B}$: ΔUA2S-Gal-NAc4S; $2S6S_{CS\text{-}D}$: ΔUA2S-GalNAc6S; $4S6S_{CS\text{-}E}$: ΔUA-GalNAc4S6S; $TriS_{CS}$: ΔUA2S-GalNAc4S6S), unsaturated disaccharide standards of HS (0S: ΔUA-GlcNAc; NS: ΔUA-GlcNS; 6S: ΔUA-GlcNAc6S; 2S: ΔUA2S-GlcNAc; 2SNS: ΔUA2S-GlcNS; NS6S: ΔUA-GlcNS6S; 2S6S: ΔUA2S-GlcNAc6S; TriS: ΔUA2S-GlcNS6S), and unsaturated disaccharide standard of HA ($0S_{HA}$: ΔUA-GlcNAc), where ΔUA is 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid, were

purchased from Seikagaku (Tokyo, Japan). Internal standard ΔUA2S-GlcNCOEt-6S (Heparin Disaccharide) was from Galen laboratory supplies (Middletown, CT). The saturated disaccharide standards, Gal-GlcNAc6S (NS_{KS}) and Gal6S-GlcNAc6S ($6SNS_{KS}$) for KS (from bovine corneal KS) were prepared in our laboratory using keratanase II. Actinase E was obtained from Kaken Biochemicals (Tokyo, Japan). Chondroitin lyase ABC from *Proteus vulgaris* and chondroitin lyase ACII from *Arthrobacter aurescens*, and Keratanase from *Pseudomonas sp.* were obtained from Seikagaku (Tokyo, Japan). Recombinant *Flavobacterial* heparin lyases I, II, and III were expressed in our laboratory using *Escherichia coli* strains provided by Jian Liu (College of Pharmacy, University of North Carolina, Chapel Hill). 2-Aminoacridone (AMAC) and sodium cyanoborohydride ($NaCNBH_3$) were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals were of HPLC grade. Vivapure Q Mini H strong anion exchange spin columns were from Sartoriou Stedim Biotech (Bohemia, NY). Spectra/Por 6 dialysis tubing (molecular weight cut off: MWCO = 8 kDa) was from spectrum labs (Rancho Dominguez, CA).

Preparation of chicken eggshell components

After broken strength determination using an FHK testing machine (Fujihara Co., Tokyo, Japan), each egg was broken in half, the egg content was discarded, and eggshell was immediately washed extensively with distilled water then rinsed into 5% EDTA for 20 min to facilitate mechanical removal of cuticle and membranes. The calcified eggshell was dried at room temperature, and then the thickness was determined and the eggshell was powdered. The membranes were freeze-dried and powdered using dry ice for GAG recovery.

The calcified shell matrices were extracted from the eggshell. Briefly, the eggshell powder was decalcified by stirring with excess of 10% acetic acid at 4°C for 28 h; the mixture was centrifuged at 23,500 × g for 16 min. The deposit was extensively washed with distilled water and again centrifuged; the pellet was freeze-dried and designated as acid-insoluble matrix. The supernatant (referred to as acid-soluble matrix) was further dialyzed against a 150-times volume of distilled water at 4°C for 24 h. The sample was centrifuged at 4,500 × g for 20 min to obtain a water-insoluble matrix (deposit) and a water and acid facultative-soluble matrix (supernatant). The water-insoluble matrix was freeze-dried, and the water-soluble matrix was concentrated using a spin column (molecular weight cutoff [**MWCO**] 10 kDa) and freeze-dried. After freeze-drying, each matrix was individually weighed; the matrices from the same calcified shell were pooled and powdered using dry ice for GAG recovery.

GAG recovery from eggshell components

The membrane powder or calcified shell matrix powder was individually subjected to proteolysis at 55°C for 24 h using actinase E (0.25 mg actinase/mg dry membrane or shell matrix). After centrifugation at 6,500 × g for 20 min, the deposit was re-suspended and proteolysis performed again using the original system. Both supernatants were pooled and transferred into MWCO 3 kDa spin columns to further remove small peptides, then freeze-dried. The dried samples were each dissolved into 8 M urea containing 2% CHAPS (pH 7.8) and bound to Vivapure Q Mini H spin columns, after washing 4 times with 200 mM NaCl.; crude GAG were eluted using 16% NaCl. Finally, the crude GAG samples were desalted using a YM-10 spin column and freeze-dried.

Disaccharide preparation of GAG in eggshell components

The crude GAG in the membranes or calcified shells were individually and completely depolymerized using polysaccharide lyases and KS hydrolase. To prepare disaccharides of CS and HA, chondroitin lyase ABC (500 mU) and chondroitin lyase ACII (50 mU) in 0.1% BSA were added to ∼100 μg crude GAG in buffer of 100 mM ammonium acetate containing 10 mM $CaCl_2$ (pH 7.5; final volume 60 μL). These mixtures were incubated at 36°C for 15 h. To prepare disaccharides of HS and KS, heparin lyase I (500 mU), II (500 mU), and III (500 mU) in 25 mM Tris-HCl, 500 mM NaCl, 300 mM imidazole buffer (pH 7.5) were added to ∼100 μg crude GAG (final volume 60 μL). After incubation at 36°C for 5 h, keratanase (30 mU) in 0.3% BSA and 120 mM Tris-HCl buffer was added (pH 7.5; final volume 90 μL) and incubated at 36°C for additional 12 h. After this incubation, 500 nanogram of internal standard disaccharide was added to each specimen, then mixtures were boiled to inactivate the enzymes at 100°C for 2 min and centrifuged with 14,500 × g for 15 min; the supernatants were first dried in a rotary concentrator and then freeze-dried.

Derivation of unsaturated disaccharides with AMAC

The freeze-dried biological sample containing both GAG-derived disaccharides and internal standard disaccharide or a mixture of unsaturated disaccharide standards was added to 10 μL 0.1 M AMAC solution in acetic acid (AcOH)/dimethyl sulfoxide (DMSO) (3:17, v/v) and mixed by transient votexing and left standing at room temperature for 30 min. Next, 10 μL of one M $NaBH_3CN$ was added in the reaction mixture and incubated at 45°C for 4.5 h. Finally, the AMAC-tagged disaccharide mixtures were centrifuged with 14,500 × g

for 15 min, and the supernatants were used for LC-MS analysis.

Disaccharide analyses using LC-MS

LC-MS analyses were performed on an Agilent 1200 LC/MSD Instrument (Agilent Technologies, Inc., Wilmington, DE) equipped with a 6300 ion-trap and a binary pump equipped with a high-pressure cell. The column used was an Agilent Poroshell 120 EC-C18 column (3.0 mm × 150 mm, 2.7 μm, Agilent Technologies, Inc., Wilmington, DE) at 45°C.

For dual ammonium acetate and methanol gradient, eluent A was 50 mM ammonium acetate solution and eluent B was methanol. Solution A and 5% solution B was flowed (150 μL/min) through the column followed by linear gradients of 5 to 17% solution B from zero to 20 min, 17 to 40% solution B from 20 to 30 min, and 40 to 100% solution B from 30 to 31 min.

The column effluent entered the ESI-MS source for continuous detection by MS. The electro-spray 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 temperature of 350°C to obtain the maximum abundance of the ions in a full-scan spectrum (350 to 900 Da). Nitrogen (8 L/min, 40 psi) was used as a drying and nebulizing gas.

GAG composition and disaccharide analysis of the chicken eggshell components were determined based on mass spectrometry total ion chromatography (**TIC**) and extracted ion chromatography (**EIC**), and compared to the retention time, molecular weight, and ratio of mass to charge (**m/z**) of each disaccharide standard.

Absolute quantification analysis of AMAC-labeled HS, CS/DS, and HA disaccharides was performed using calibration curves established by separation of increasing amounts of disaccharide standards (0.1, 0.5, 1, 5, 10, 20, 50, and 100 ng/each). Linearity was assessed based on the amount of disaccharide and peak intensity in EIC. Quantitative analysis of AMAC-labeled KS disaccharides in the samples was based on the KS-disaccharides in the bovine corneal KS acquired by keratanase II digestion (Liu et al., 2014). It was reported that the molar proportion of $6SNS_{KS}/NS_{KS}$ was 38: 53 (Plaas et al., 2001). According to these different concentrations of bovine corneal KS disaccharides analyzed by LC-MS, the KS disaccharides in the samples were analyzed and quantified.

Relative quantification analysis of AMAC-labeled HS, CS, HA, and KS disaccharides was performed based on the ratio of disaccharide peak area to the peak area of internal standard disaccharide.

Statistical analysis

The software of bivariate correlations in SPSS 19.0 was used to determine the correlations among several variables. The results were expressed as Pearson correlation coefficients. In certain cases of this paper, some variants can influence other variants in only one way, such as components in membranes can influence components or properties of calcified shell in only one way, but not mutually, so a one-tailed test of significance (half of two-tailed significance) was adopted.

RESULTS

Population characteristics of glycosaminoglycan disaccharides in chicken eggshells

GAG compositions and disaccharides in the chicken eggshell components were determined. As previous research showed (Liu et al., 2014), the occurrence of all 4 groups of GAG was detected not only in shell membranes but also in calcified shells (Figures 1 and 2); furthermore, these GAG may be the constitutive polysaccharide of shell membranes and calcified shells (population frequencies were 100%) (Tables 1 and 2).

The KS in chicken eggs contained only NS_{KS} (Gal-GlcNAc6S) and lacked $6SNS_{KS}$ (Gal6S-GlcNAc6S) (Liu et al., 2014), and the NS_{KS} was present in all detected shell membranes and calcified shells (population frequency was 28/28) (Table 1). The $4S6S_{CS\text{-}E}$ and $2S_{CS}$ disaccharides are absent from the CS found in both membranes and calcified shells (0/28), while $6S_{CS\text{-}C}$, $4S_{CS\text{-}A}$, and $0S_{CS\text{-}0}$ disaccharides were constituent in both shell components (28/28), and the $4S_{CS\text{-}A}$ disaccharide was detected with isomers (Figures 1 and 2). The $TriS_{CS}$ and $2S6S_{CS\text{-}D}$ were undetectable in membranes but they were present at low levels in calcified shells, respectively (1/28 and 6/28). The $2S4S_{CS\text{-}B}$ was detected in shell membranes at a low frequency (1/28) and in calcified shells with higher frequency (18/28) (Table 1). The HS, NS6S, NS, 6S, and 0S disaccharides were detected in calcified shells (Figure 2), while none of these HS disaccharide isomers was present in membranes (Figure 1). The 2S6S was absent from both membranes and calcified shells (0/28) (Figures 1 and 2). The HS TriS, NS6S, NS2S, NS, 2S6S, 6S, 2S, and 0S disaccharides were found in calcified shells with high frequency (28/28) (Table 2). In membranes both HS NS and 0S were present at high frequency (28/28), 2S was undetectable (0/28) (Figure 1), NS6S and 6S were detectable with low frequency (5/28), and TriS and NS2S were rarely present (1/28) (Table 2).

In the shell membranes, HA was the most plentiful GAG component (703.52 ± 194.49 μg per gram of membrane), followed by CS, KS, and HS (Tables 1 and 2). In the calcified shell, CS was the most abundant GAG (248.26 ± 54.55 μg per gram of calcified shell), followed by HS and HA, and KS, present in only trace amounts (0.61 ± 0.26 μg per gram of calcified shell) (Table 1). Of the CS disaccharides in shell membranes and calcified shell, the most plentiful was $4S_{CS\text{-}A}$, followed by

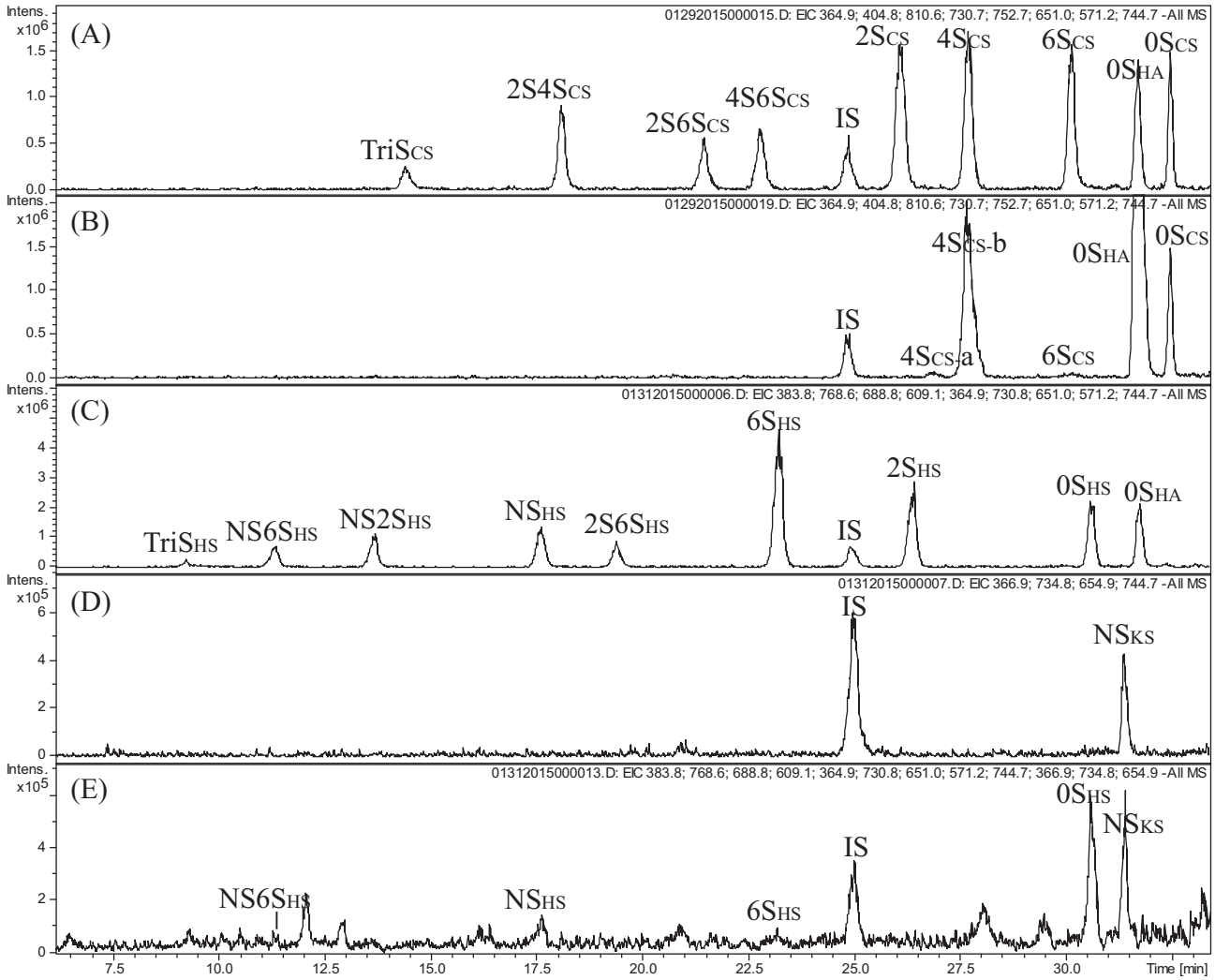

Figure 1. Glycosaminoglycan disaccharides in shell membranes. IS represents internal reference standard (ΔUA-2S-GlcNCOEt6S); A. CS/DS/HA disaccharide standards; B. GAG treated by chondroitin lyase ABC (the peaks marked –a and –b have the same molecular weight and m/z, which might be isomers); C. HS /HA disaccharide standards; D. KS disaccharide standards; E. GAGs treated by both heparin lyases and keratanase.

$0S_{CS}$ (Table 1). The most abundant HS disaccharide in both eggshell components was 0S. The HS NS was the second major component in the shell membranes, and TriS and NS were the next major components in the calcified shells (Table 2).

Furthermore, based on the analysis of correlations among molar contents of various disaccharides of specific GAG, there were very significant correlations among the CS disaccharides of the shell membranes (except $2S4S_{CS\text{-}B}$) (Table A1), among HS disaccharides of the shell membranes (except TriS) (Table A2), and among HS disaccharides of the calcified shells (except one of the 2S isomers) (Table A3). There was almost no correlation among CS disaccharides in the calcified shells (Table A4). The results suggest that CS disaccharide composition of membranes, and the HS composition of both shell components are very similar in each eggshell. There is a strong linkage among the above disaccharides in a given eggshell or even in the same polysaccharide molecule. In contrast, there is significant heterogeneity in the disaccharide composition of CS among different calcified shells. These results may provide important information for poultry breeding purposes.

Influences of GAG and GAG disaccharide composition in the shell membranes on eggshell properties and matrices

The influences of GAG and their disaccharide composition of high frequency (population frequency >50%) were determined (Table 3). Among 4 groups of GAG found in the shell membrane, both HA and KS showed significant influence on egg shape index (HA: $r = 0.335$, 1-tailed $P = 0.047$; NS_{KS}: $r = -0.334$, 1-tailed $P = 0.048$). CS showed significant influence; $4S_{CS\text{-}A}$ showed significant influence on the eggshell strength ($r = -0.346$, 1-tailed $P = 0.042$); $6S_{CS\text{-}C}$ showed significant influence on the acid insoluble matrix in

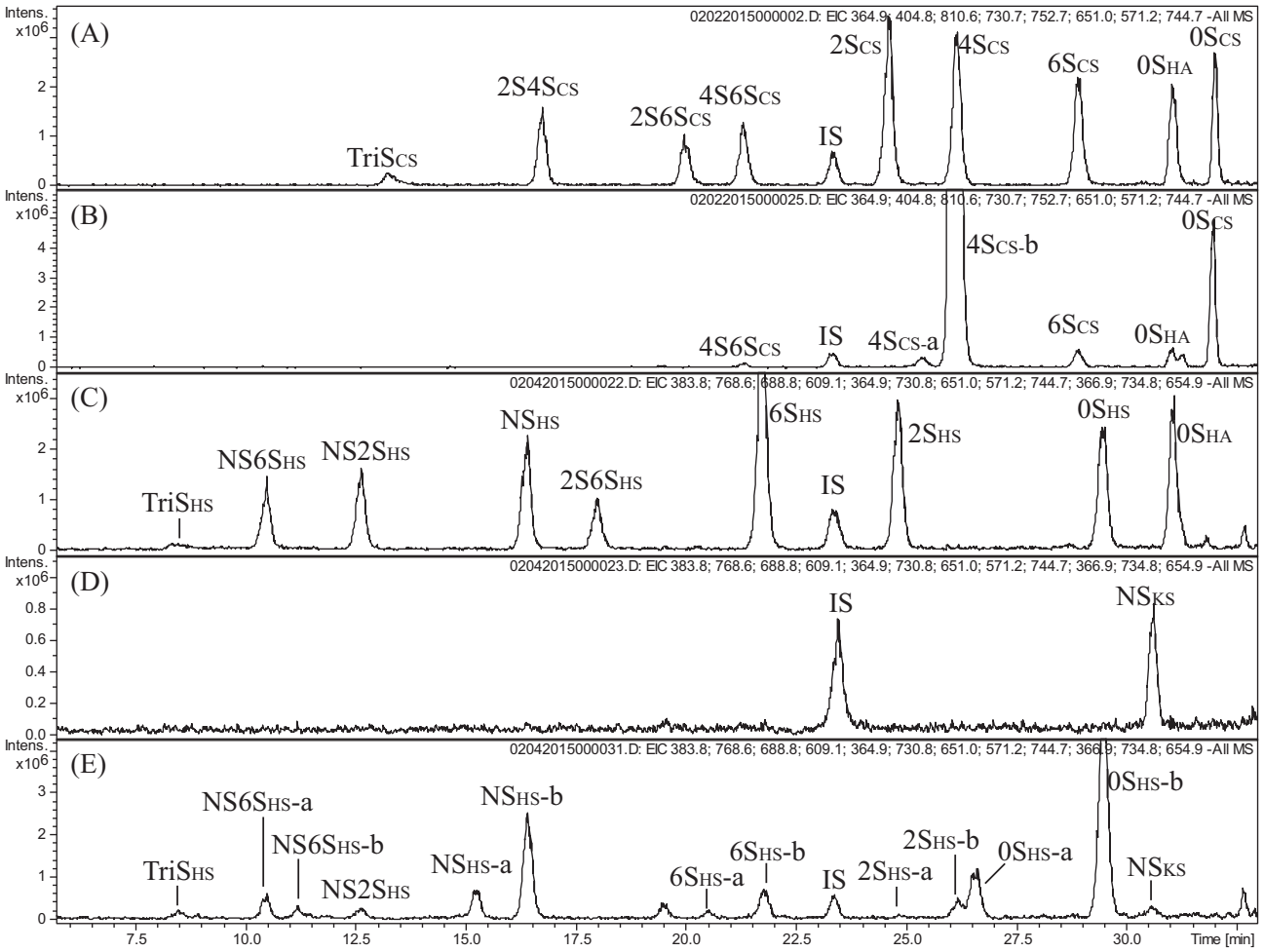

Figure 2. Glycosaminoglycan disaccharides in calcified shells. IS represents internal reference standard (ΔUA-2S-GlcNCOEt6S); A. CS/ HA disaccharide standards; B. GAG treated by chondroitin lyase ABC (the peaks marked –a and –b have the same molecular weight and m/z, which might be isomers); C. HS/ HA disaccharide standards; D. KS disaccharide standards; E. GAGs treated by both heparin lyases and keratanase (the peaks marked –a and –b have the same molecular weight and m/z, which might be isomers).

Table 1. Population characterizations of glycosaminoglycan (HA/KS/CS) fine structures in chicken eggshell.

		HA	NS_{KS}	TriS	$2S4S_{CS\text{-}B}$	$2S6S_{CS\text{-}D}$	$4S6S_{CS\text{-}E}$	$2S_{CS}$	$4S_{CSA}$	$6S_{CS\text{-}C}$	$0S_{CS\text{-}0}$	Total CS
Shell membrane	P.F.*	28/28	28/28	ND	1/28	ND**	ND	ND	28/28	28/28	28/28	28/28
	Mass***	703.5 ± 194.5	45.3 ± 29.2	ND	0.21	ND	ND	ND	45.1 ± 36.5	2.1 ± 1.2	17.9 ± 7.3	66.3 ± 43.0
Calcified shell	P.F.	28/28	28/28	1/28	18/28	6/28	ND	ND	28/28	28/28	28/28	28/28
	Mass	4.6 ± 1.8	0.6 ± 0.3	0.1	0.1 ± 0.10	0.9 ± 0.4	ND	ND	226.6 ± 51.8	1.7 ± 0.6	17.9 ± 5.1	248.3 ± 54.6

*P.F. Population frequency.
**ND: Not detectable.
***Mass: Mass contents were expressed as micrograms of disaccharide or GAG in per gram of membrane or calcified shell, average contents were expressed as mean ± standard deviation, and disaccharide contents were averaged based on the size of detectable samples instead of the size of whole population.

calcified shell (r = 0.369, 1-tailed $P = 0.046$); and $0S_{CS\text{-}0}$ showed significant influence on the whole matrix in calcified shell (r = 0.428, 1-tailed $P = 0.024$), eggshell strength (r = −0.401, 1-tailed $P = 0.021$), and calcified shell thickness (r = −0.371, 1-tailed $P = 0.031$). Total CS influenced the whole matrix in the calcified shells (r = 0.377, 1-tailed $P = 0.042$) and eggshell strength (r = −0.369, 1-tailed $P = 0.032$). Finally, HS showed no significant influence on the organic components and properties of calcified shells (1-tailed $P > 0.05$).

Correlations of GAG and GAG disaccharide composition on calcified shells and eggshell matrices

The influence of GAG and disaccharides of high frequency (population frequency >50%) in the calcified shells was next determined.

The significant correlations were detected between shell matrices and GAG or disaccharides (Table 4). The acid insoluble matrix showed significant correlations with HA (r = −0.575, 1-tailed $P = 0.002$), NS_{KS}

Table 2. Population characterizations of glycosaminoglycan (HS) fine structures in chicken eggshell.

		TriS	NS6S	NS2S	NS	2S6S	6S	2S	0S	Total HS
Shell membrane	P.F.*	1/28	5/28	1/28	28/28	ND**	5/28	ND	28/28	28/28
	Mass***	4.3	2.3 ± 1.4	2.9	4.3 ± 1.6	ND	0.7 ± 0.3	ND	14.8 ± 4.4	20.0 ± 6.8
Calcified shell	P.F.*	28/28	28/28	28/28	28/28	ND	28/28	28/28	28/28	28/28
	Mass***	4.0 ± 1.0	1.9 ± 0.5	0.4 ± 0.1	3.6 ± 0.9	ND	0.5 ± 0.1	0.5 ± 0.1	6.7 ± 1.8	17.3 ± 4.1

*P.F. Population frequency.
**ND: Not detectable.
***Mass: Mass contents were expressed as micrograms of disaccharide or GAG in per gram of membrane or calcified shell, average contents were expressed as mean ± standard deviation, and disaccharide contents were averaged based on the size of detectable samples instead of the size of whole population.

Table 3. Correlations between GAG in the membrane and eggshell matrices or eggshell parameters.

GAG	Acid insoluble	Water insoluble	Soluble	Matrix	Egg weight	Egg length	Egg width	Egg shape	Strength	Thickness
HA	0.286	−0.237	−0.284	0.187	−0.089	0.112	−0.207	0.335*	−0.173	0.063
NS_{KS}	−0.103	0.115	−0.103	−0.075	0.120	−0.112	0.211	−0.334*	0.011	0.024
$4S_{CS-A}$	0.333	0.137	−0.026	0.346	−0.260	−0.273	−0.261	−0.028	−0.346*	−0.163
$6S_{CS-C}$	0.369*	−0.208	−0.130	0.276	−0.114	−0.124	−0.083	−0.050	−0.153	0.033
$0S_{CS-O}$	0.352	0.227	0.195	0.428*	−0.228	−0.257	−0.190	−0.083	−0.401*	−0.371*
Total CS	0.355	0.150	0.007	0.377*	−0.265	−0.282	−0.259	−0.040	−0.369*	−0.202
NS_{HS}	0.231	0.153	0.004	0.261	−0.058	−0.179	−0.063	−0.129	−0.175	0.007
$0S_{HS}$	0.339	0.022	−0.062	0.327	−0.014	−0.147	−0.005	−0.152	−0.254	−0.068
Total HS	0.237	0.129	−0.116	0.245	−0.021	−0.175	−0.009	−0.179	−0.240	−0.007
Total GAGs	0.311	−0.158	−0.264	0.233	−0.114	0.024	−0.203	0.235	−0.227	0.019

*Means the significance was at the level <0.05 in one-tailed.

Table 4. Correlations between GAG or disaccharides in calcified shell and eggshell matrices or eggshell parameters.

GAG	Acid insoluble	Water insoluble	Soluble	Matrix	Egg weight	Egg length	Egg width	Shape index	Strength	Thickness
HA	−0.575**	−0.127	−0.209	−0.588**	−0.169	−0.136	−0.100	−0.048	−0.301	−0.223
NS_{KS}	−0.377*	−0.223	−0.247	−0.428*	−0.144	0.048	−0.177	0.252	−0.010	0.021
$2S4S_{CS-B}$	0.002	0.263	−0.127	0.045	−0.251	−0.223	−0.308	0.089	0.222	0.123
$4S_{CS-A}$	0.039	0.314	0.177	0.125	−0.414*	−0.297	−0.482**	0.194	−0.019	−0.160
$6S_{CS-C}$	0.391*	−0.364*	−0.078	0.276	−0.074	0.050	−0.086	0.150	0.139	0.156
$0S_{CS-O}$	0.235	0.302	0.234	0.312	−0.215	−0.191	−0.207	0.010	−0.204	−0.197
Total CS	0.068	0.325	0.194	0.156	−0.414*	−0.300	−0.479**	0.187	−0.039	−0.169
$TriS_{HS}$	0.298	−0.087	0.048	0.264	0.234	0.377*	0.128	0.297	−0.146	−0.184
$NS6S_{HS}$	−0.003	0.093	0.390*	0.062	−0.012	0.019	0.011	0.011	−0.076	−0.045
$NS2S_{HS}$	0.022	0.137	0.255	0.079	−0.164	−0.066	−0.209	0.156	−0.046	−0.084
NS_{HS}	0.162	0.180	0.358*	0.231	−0.014	0.058	−0.028	0.099	0.119	0.068
$6S_{HS}$	0.012	−0.284	0.119	−0.037	0.081	0.310	−0.003	0.364*	−0.073	0.045
$2S_{HS}$	−0.137	0.002	0.129	−0.112	−0.119	0.061	−0.134	0.215	0.071	−0.068
$0S_{HS}$	0.023	0.018	0.320	0.061	0.047	0.166	−0.006	0.202	0.220	0.227
Total HS	0.102	0.039	0.307	0.138	0.056	0.177	0.004	0.203	0.102	0.088
Total GAGs	0.050	0.313	0.203	0.138	−0.405*	−0.283	−0.470**	0.196	−0.041	−0.166

*Means the significance was at the level <0.05 in one-tailed.
**Means the significance was at the level <0.01 in one-tailed.

(r = −0.377, 1-tailed $P = 0.035$), and $6S_{CS-C}$ (r = 0.391, 1-tailed $P = 0.030$). The water insoluble matrix showed significant correlation with $6S_{CS-C}$ (r = −0.364, 1-tailed $P = 0.040$). The water and acid facultative-soluble matrix showed significant correlations with $NS6S_{HS}$ (r = 0.390, 1-tailed $P = 0.030$) and NS_{HS} (r = 0.358, 1-tailed $P = 0.043$). Finally, the whole matrix showed significant correlations with HA (r = −0.588, 1-tailed $P = 0.001$) and NS_{KS} (r = −0.428, 1-tailed $P = 0.018$). It is notable that $4S_{CS-A}$, the most abundant disaccharide, showed no significant correlations with any matrices (1-tailed $P > 0.05$).

Both egg weight and egg width showed significant correlations with $4S_{CS-A}$ (egg weight: r = −0.414, 1-tailed $P = 0.022$; egg width: r = −0.482, 1-tailed $P = 0.009$), CS/DS (egg weight: r = −0.414, 1-tailed $P = 0.022$; egg width: r = −0.479, 1-tailed $P = 0.009$), and the whole GAG (egg weight: r = −0.405, 1-tailed $P = 0.025$; egg width: r = −0.470, 1-tailed $P = 0.010$) (Table 3). These results may be because $4S_{CS-A}$ was the predominant component in CS and even in the total GAG found in the calcified shells (Table 1). Additionally, the significant correlation was present between egg length and HS TriS (r = 0.377, 1-tailed $P = 0.035$), and between egg shape index and $6S_{HS}$ (r = 0.364, 1-tailed $P = 0.040$) (Table 3). As a whole, no GAG and disaccharides in the calcified shells showed significant correlations with eggshell strength and thickness (Table 4).

DISCUSSION

In the present research, we set special rules to exclude some eggs of extreme sizes and shapes. Furthermore, it is known that eggshell broken strength is highly associated with thickness (in the present research, eggshell strength [3.715 ± 0.844 kgf] ranged 2.36 to 5.45 kgf, and calcified shell thickness [0.322 ± 0.030 mm] ranged 0.233 to 0.365 mm. The correlation between both variants was r = 0.717, 1-tailed $P = 0.000$). At least 6 samples were determined in each area, such as blunt, sharp, and equator areas of eggshells, and the thickness difference was restricted under the criterion of 0.03 mm to keep the thickness homogeneity in the same eggshell. Practically, about 10% of eggshells were selected as specimens according to these criteria. Among the losing eggshells, the majority failed based on the thickness criterion, suggesting a significant heterogeneity of thickness frequently occurs in the same calcified eggshell.

In previous research, some eggs laid by free range White Leghorn chickens were separated into various components, then each specimen of the same component was pooled. The previously reported GAG composition of the chicken egg components (Liu et al., 2014) showed the occurrence of all 4 groups of GAG types in both shell membranes and calcified shells. The present results show that all 4 groups of GAG may be intrinsic sugar components, not only of shell membranes but also of calcified shells (population frequencies were 100%) (Tables 1 and 2). Three CS disaccharides, $4S_{CS\text{-}A}$, $6S_{CS\text{-}C}$, and $0S_{CS\text{-}0}$, were detected as constituent components in both membranes and calcified shells (population frequencies were 100%); the $2S4S_{CS\text{-}B}$ showed an intermediate frequency and $TriS_{CS}$ with very low frequency in the calcified shells (Table 1). These results agree well with previously published results (Liu et al., 2014) in which $4S_{CS\text{-}A}$, $6S_{CS\text{-}C}$, and $0S_{CS\text{-}0}$ were detected with higher constituent proportions in both shell membranes and calcified shells, and that calcified shells also contained lower proportions of $TriS_{CS}$ and $2S4S_{CS\text{-}B}$. However, significant differences also were observed between the two studies; the presence of $4S6S_{CS\text{-}E}$ and absence of $2S6S_{CS\text{-}D}$ in calcified shells were detected in the previous study (Liu et al., 2014) but the present study of calcified shells showed $4S6S_{CS\text{-}E}$ was not detectable and $2S6S_{CS\text{-}D}$ was present with a low population frequency (Table 1). Since the methods used in both studies were nearly identical, these differences might be attributed to different egg sources. Examination of the disaccharide composition of HS in both studies showed that 2S6S was undetectable in either shell membranes or calcified shells, and the other 6 disaccharides were all detected, even as constituent components in the calcified shells (Table 2) (Liu et al., 2014). However, in membranes — although the population frequencies of the 4 HS disaccharides, NS2S, NS, 6S, and 0S, in the present research are in agreement with the constituent proportions of the same components in the previous study (Table 1) (Liu et al., 2014) — both HS TriS and NS6S were unexpectedly detected with low population frequencies in the present study (Table 1). These differences might also be the result of different egg sources. Finally, in the present study, disaccharide $4S_{CS\text{-}A}$ in both membranes and calcified shells, and disaccharides of HS in calcified shells, such as NS6S, NS, 6S, 2S, and 0S, were detected as isomers (Table 2); however, these disaccharide isomers were never observed in the previous study (Liu et al., 2014).

Uronic acid is a constituent sugar of all GAG with the exception of KS, and it is well known that uronic acid is in both eggshell membranes and in calcified shells (Bronsch and Diamantstein, 1965). Since GAG are negatively charged macromolecules with plenty of carboxyl and/or sulfo groups, GAG and their parent proteoglycans are thought to alter precipitation of ions during biomineralization, performing a role in ensuring eggshell quality. It was reported that the feedstuff with manganese supplementation can promote the synthesis of GAG in membranes for improved eggshells (Xiao et al., 2014). Using eggshell membranes as a substrate, CS proteoglycans from calcified shells in vitro can cause a concentration-dependent change in calcite morphology (Carrino et al. 1996). Based on colorimetric analysis, it was reported that in shell membranes GAG were highly associated with shell strength. Galactose, a constituent of KS, shows a significant correlation with eggshell strength and uronic acid, a constituent sugar of all GAG, excluding KS, weakly correlates to shell strength (Ha et al. 2007). In present membranes, there were no significant correlations among eggshell broken strength and total GAG content (r = −0.227, 1-tailed $P = 0.132$), and KS (r = 0.011, 1-tailed $P = 0.478$) (Table 3) and GAG excluding KS (r = −0.222, 1-tailed $P = 0.132$). The differences between the previous and current study may be the results of different methods used as well as improved analytical sensitivity. However, present results showed that $0S_{CS}$ had significant correlations with eggshell strength (r = −0.401, 1-tailed $P = 0.021$), and with calcified shell thickness (r = −0.371, 1-tailed $P = 0.031$) (Table 2). Total CS showed significant influence on eggshell strength (r = −0.369, 1-tailed $P = 0.032$) (Table 2). It is known that the mammillary knobs (also named calcium reserve assemblies) are the sites for crystal nucleation and the initial phases of calcium deposition, and the mammillary knobs are built externally on the shell membranes (Stemberger et al., 1977), so it might be possible that the CS GAG or its disaccharide $0S_{CS}$ negatively controls eggshell quality by regulating the formation of mammillary knobs, and they can be used as an assisted marker for breeding for expected eggshell quality.

In the calcified shells, it has been speculated that KS in the mammillary region might perform a role in the nucleation of the first calcite crystals (Arias et al., 1992; Fernandez et al., 2001) and CS type-B might regulate the crystal growth and orientation of the later forming eggshell (Fernandez et al., 1997; Fernandez et al., 2001). Furthermore, it has been reported that there is a

significant correlation between the uronic acid content in calcified shell and eggshell strength (Bronsch and Diamantstein, 1965). However, in the current study, the GAG excluding KS (a marker of uronic acid content) in calcified shell showed no significant correlation between eggshell strength ($r = -0.038$, 1-tailed $P = 0.430$). The different analysis methods used in both studies might partially account for the above difference. Moreover, except for $6S_{HS}$, which showed significant correlation with egg shape (Table 4), other GAG or their disaccharide composition in calcified shells showed no significant correlation with eggshell strength, calcified shell thickness, or egg shape (Table 4). These results were surprising and beyond expectation. It is possible that GAG or disaccharides might perform multiple functions in the eggshell mineralization process, such as organic matrix organization, mineral deposition, and crystal growth, but these functions may not be achieved in a concentration-dependent manner.

CONCLUSIONS

All four groups of GAG were detected as constitutive polysaccharides in not only shell membranes but also in calcified shells. Moreover, HA was the most plentiful GAG in membranes, and CS was the most abundant in calcified shell. The CS disaccharides $6S_{CS\text{-}C}$, $4S_{CS\text{-}A}$, and $0S_{CS\text{-}0}$ were constitutive in both shell membranes and calcified shells, $4S6S_{CS\text{-}E}$ and $2S_{CS}$ were undetectable in both shell components, $2S4S_{CS\text{-}B}$ was detectable in membranes with a low frequency, and $TriS_{CS}$, $2S4S_{CS\text{-}B}$, and $2S6S_{CS\text{-}D}$ were detected in calcified shells with low frequency. The HS disaccharides TriS, NS6S, NS2S, NS, 2S6S, 6S, 2S, and 0S were almost constitutive in calcified shells except 2S6S, which was absent. In membranes, both NS and 0S were constitutive; TriS, NS2S, NS6S, and 6S were detectable with low frequency; and both $2S6S_{HS}$ and $2S_{HS}$ were undetectable.

The disaccharide constitutions of membrane CS, or of membrane HS, or of calcified shell HS, were very similar in each eggshell; however, the constitution of CS disaccharides in each calcified shell showed significant heterogeneity.

The correlation analysis of GAGs in the membrane showed that the HS and its disaccharides showed no significant influence on properties of calcified shells. HA and NS_{KS} showed significant influence on egg shape index. The $4S_{CS\text{-}A}$ showed significant influence on the eggshell strength and the $0S_{CS\text{-}0}$ had significant influence on the whole matrix content, eggshell strength, and calcified shell thickness. Total CS can influence the whole matrix, and eggshell strength. Total CS might regulate the formation of mammillary knobs in concentration-dependent form and then control eggshell quality.

Finally, almost no GAG and no GAG disaccharides in the calcified shells showed significant correlations with eggshell strength and thickness. During the eggshell mineralization process, GAG might perform multiple functions, such as organic matrix organization, mineral deposition, and crystal growth, but these functions are not concentration-dependent.

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APPENDIX

Table A1 Correlations between molar contents of disaccharides of CS in shell membranes.

Disaccharides	$2S4S_{CS\text{-}B}$	$4S_{CSA}$-a	$4S_{CSA}$-b	$6S_{CS\text{-}C}$	$0S_{CS\text{-}O}$
$2S4S_{CS\text{-}B}$	1	0.251	0.417*	0.335*	0.147
$4S_{CSA}$-a	0.251	1	0.752**	0.581**	0.712**
$4S_{CSA}$-b	0.417*	0.752**	1	0.482**	0.697**
$6S_{CS\text{-}C}$	0.335*	0.581**	0.482**	1	0.562**
$0S_{CS\text{-}O}$	0.147	0.712**	0.697**	0.562**	1

Note: 1) correlations meant Pearson Correlations; 2) *meant the significance was at the level < 0.05 in one-tailed, **meant the significance was at the level < 0.01 in one-tailed

Table A2 Correlations between molar contents of disaccharides of HS in shell membranes.

Disaccharides	$TriS_{HS}$	$NS6S_{HS}$	$NS2S_{HS}$	NS_{HS}	$6S_{HS}$	$0S_{HS}$
$TriS_{HS}$	1	0.335*	− 0.040	0.032	− 0.090	− 0.098
$NS6S_{HS}$	0.335*	1	0.761**	0.677**	0.372*	0.435*
$NS2S_{HS}$	− 0.040	0.761**	1	0.584**	0.527**	0.354*
NS_{HS}	0.032	0.677**	0.584**	1	0.534**	0.661**
$6S_{HS}$	− 0.090	0.372*	0.527**	0.534**	1	0.471**
$0S_{HS}$	− 0.098	0.435*	0.354*	0.661**	0.471**	1

Notes: 1) correlations meant Pearson Correlations; 2) *meant the significance was at the level < 0.05 in one-tailed, **meant the significance was at the level <0.01 in one-tailed.

Table A3 Correlations between molar contents of disaccharides of CS in calcified shells.

Disaccharides	$TriS_{CS}$	$2S4S_{CS\text{-}B}$	$2S6S_{CS\text{-}D}$	$4S_{CSA}$-a	$4S_{CSA}$-b	$6S_{CS\text{-}C}$	$0S_{CS\text{-}O}$
$TriS_{CS}$	1	− 0.054	− 0.096	− 0.201	− 0.166	− 0.221	− 0.060
$2S4S_{CS\text{-}B}$	− 0.054	1	− 0.106	0.116	0.676**	0.278	0.335
$2S6S_{CS\text{-}D}$	− 0.096	− 0.106	1	− 0.124	0.021	0.080	0.074
$4S_{CSA}$-a	− 0.201	0.116	− 0.124	1	0.274	0.284	− 0.323
$4S_{CSA}$-b	− 0.166	0.676**	0.021	0.274	1	0.250	0.433*
$6S_{CS\text{-}C}$	− 0.221	0.278	0.080	0.284	0.250	1	− 0.016
$0S_{CS\text{-}O}$	− 0.060	0.335	0.074	− 0.323	0.433*	− 0.016	1

Notes: 1) correlations meant Pearson Correlations; 2) *meant the significance was at the level < 0.05 in one-tailed, **meant the significance was at the level <0.01 in one-tailed.

Table A4 Correlations between molar contents of disaccharides of HS in calcified shells.

Disaccharides	$TriS_{HS}$	$NS6S_{HS}$-a	$NS6S_{HS}$-b	$NS2S_{HS}$	NS_{HS}-a	NS_{HS}-b	$6S_{HS}$-a	$6S_{HS}$-b	$2S_{HS}$-a	$2S_{HS}$-b	$0S_{HS}$-a	$0S_{HS}$-b
$TriS_{HS}$	1	0.546**	0.452*	0.618**	0.608**	0.601**	0.599**	0.590**	0.288	0.534**	0.492**	0.527**
$NS6S_{HS}$-a	0.546**	1	0.743**	0.718**	0.849**	0.885**	0.770**	0.773**	0.076	0.742**	0.877**	0.837**
$NS6S_{HS}$-b	0.452*	0.743**	1	0.726**	0.803**	0.751**	0.713**	0.617**	0.201	0.562**	0.649**	0.642**
$NS2S_{HS}$	0.618**	0.718**	0.726**	1	0.750**	0.756**	0.664**	0.605**	0.435*	0.695**	0.672**	0.672**
NS_{HS}-a	0.608**	0.849**	0.803**	0.750**	1	0.951**	0.819**	0.689**	0.242	0.620**	0.838**	0.889**
NS_{HS}-b	0.601**	0.885**	0.751**	0.756**	0.951**	1	0.800**	0.766**	0.226	0.695**	0.913**	0.938**
$6S_{HS}$-a	0.599**	0.770**	0.713**	0.664**	0.819**	0.800**	1	0.824**	0.071	0.629**	0.789**	0.855**
$6S_{HS}$-b	0.590**	0.773**	0.617**	0.605**	0.689**	0.766**	0.824**	1	0.137	0.589**	0.792**	0.838**
$2S_{HS}$-a	0.288	0.076	0.201	0.435*	0.242	0.226	0.071	0.137	1	− 0.022	0.128	0.206
$2S_{HS}$-b	0.534**	0.742**	0.562**	0.695**	0.620**	0.695**	0.629**	0.589**	− 0.022	1	0.787**	0.659**
$0S_{HS}$-a	0.492**	0.877**	0.649**	0.672**	0.838**	0.913**	0.789**	0.792**	0.128	0.787**	1	0.938**
$0S_{HS}$-b	0.527**	0.837**	0.642**	0.672**	0.889**	0.938**	0.855**	0.838**	0.206	0.659**	0.938**	1

Notes: 1) correlations meant Pearson Correlations; 2) *meant the significance was at the level < 0.05 in one-tailed, **meant the significance was at the level <0.01 in one-tailed.